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A STUDY OF HALOPHILIC BACTERIA IN COASTAL REGIONS OF DAKSHINA KANNADA, KARNATAKA, INDIA

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1.0 INTRODUCTION

Moderate ecological conditions which include normal temperatures, pH values, and oxygen availability levels, constitute a typical home to life. Extremophilic organisms are those that live amid challenging circumstances that are inhospitable to the great majority of other living forms (Shah and Dey, 2024). Extremophiles find extensive uses in the food, pharmaceutical and textile sectors. Halophiles are one type of such extremophile (Chen and Jiang, 2018).

Halophiles live in extremely salinized conditions, between 0.8 M and 8 M NaCl (w/v) (Oren, 2002b). The groups of halophiles that researchers have identified are: halotolerant, slight halophiles which are found growing at 0.2 M to 0.5 M NaCl, moderate halophiles which are found growing at 0.5 M to 2.5 M NaCl, borderline extreme halophiles which are found at 2.5 M to 4 M NaCl, extreme halophiles which are found growing at 4 M to 5.9 M NaCl, and non-halophiles which are found growing below 0.2 M NaCl (Cira-Chávez *et al*., 2019). Enzymes, biosurfactants, proteins, and other biomolecules found in halophiles can withstand a variety of unfavorable physiological conditions, including elevated salinity levels, elevated temperatures, elevated pH, and dehydration (Singh and Singh, 2017). The enzyme produced by halophiles maintains its activity by making structural modifications by accumulating greater proportion of hydrophilic amino acids and fewer hydrophobic amino acids on its surface (Bolhuis *et al*., 2013). Additionally, the enzyme uses the water that is available to distribute charges at its active site (Zaccai, 2011). The unique properties of the halophilic enzymes made the researchers to gain interest in this area (Karan *et al*., 2012). There are numerous applications for halophiles in the field of biotechnology, fermented pickles, polymeric materials, biodegradation, biofuels, and so forth (Gupta *et al*., 2016; Oren, 2002b; Gomes *et al*., 2018; Chen and Jiang, 2018). Lipases, xylanases, L-asparaginase, DNases, glucose dehydrogenase, cellulases, proteases and glutaminase (Flores-Gallegos *et al*., 2019), nucleases and chitinases (Oren, 2010; Moreno *et al*., 2013; Sánchez-Porro, 2003) are some of the reported haloenzymes.

The aquatic life has evolved a remarkable resilience to harsh conditions such as temperature fluctuations, chemical exposure, and radiation. Certain environmental constraints, such as the availability of sunshine and nutrition, temperature and pressure circumstances, and salinity levels that exceed what is normally considered tolerable, can cause proteins to undergo proteolysis and lose their original shape (Thombre and Oke, 2015; Nath and Bharathi, 2011). The biochemical characteristics of these proteins showed that they have a high pH and high operation temperature, which may qualify them for application in industrial environments (Neifar *et al*., 2019; Bang *et al*., 2018).

The soil region surrounding a root of a plant which is inhabited by wide variety of the microorganisms is known as the rhizosphere (Choudhary *et al*., 2016). Specific compounds released by plant roots attract microorganisms like fungus and bacteria to help sustain the rhizosphere's nutrient supply. The bacteria living in rhizosphere provide medicinal and bioremediation characteristics (Hayat *et al*., 2010). Under circumstances of stress from salt on brinjal, tomato, and potato plants, the halophiles such as, *Brevibacillus agri*, *Brevibacillus fluminis* and *Bacillus paralicheniformis* have reported to enhance plant growth (Goswami *et al*., 2019).

The microorganisms have certain physiological and biochemical parameters for their optimal growth. Thus, it is crucial to study the optimum physiological and biochemical circumstances for the optimum growth potential of the microorganisms. The halotolerant and halophilic isolates of bacteria, which belonged to the species *Oceanobacillus aidingensis, H. stenophila B. licheniformis, Bacillus haynesii* and *Halomonas pacifca* were found in the coastal region soils of Saurashtra, India. They exhibited PGPR traits and were able to withstand concentrations of salt up to 25% with optimal growth occurring between 10 - 15% NaCl (w/v); their ideal pH ranged between 6 and 8 with tolerance closer to high acids and a greater tendency towards alkalinity; and they could withstand temperatures till 45 °C, the temperature that is optimal for growth being observed at 35 °C (Reang *et al*., 2022). Microorganisms that are both autotrophic and heterotrophic were isolated from Sicily, Italy. The bacterial isolates have been identified to be *Halomonas sp., Dunaliella viridis, Salinibacter ruber, Brevibacterium sp., Dactylococcopsis salina*, and showed growth at lower and higher salinity. It was discovered that a few of the isolated strains could produce recognized bioactive compounds and had bioactivity toward human cells (Villanova *et al*., 2021).

The extracellular metallo-endopeptidase or metalloproteinase known as gelatinase hydrolyses a variety of materials, including collagen, casein, pheromone, elastin, laminin, fibronectin, and fibrinogen (Balan *et al*., 2012; Mäkinen *et al*., 1994; Mäkinen *et al*., 1989). Including food and basic biological sciences, gelatinase is extensively used in the chemical sector and medicinal fields (Balan *et al*., 2012). Search for isolates having gelatinase of high activity, having tolerance to extreme physiological conditions and cost-effective has wide range of potential applications and increasing demand (Paramashree and Nanjundaiah, 2022).

The oxidoreductase enzyme catalase degrades H_2O_2 and acts as an antioxidant. One molecule of catalase can hydrolyze a million molecules of hydrogen peroxide (Kaushal *et al*., 2018). The pH range of 4 to 11 is said to be where catalase is active (Diaz *et al*., 2012; Correia *et al*., 1994; Calera *et al*., 2000; Beers, 1961). Catalase is used in different fields such as medical, reverting malignant features, clinical diagnosis, treatment of oxidative stress, food industry in wrappers for food, systems that catalyse (Kaushal *et al.,* 2018) and oxidize glucose, production of cheese, determination of milk quality, bioremediation, hydrocarbon degradation, provider of oxygen in oxidative bioremediation, management of textile industry bleaching effluents, biocorrosion of metallic substances, and elimination of peroxide from the contact lens fluid (Youn *et al*., 1995; Beers and Sizer, 1952; Gromada and Fiedurek, 1997; Hussein, 2012).

When realistic and advantageous goals are taken into account, the advantages of using enzyme sources obtained from microbes include their rapid and inexpensive development, as well as the requirement for fewer places for large-scale production.

Marine enzyme biotechnology is the main focus of future industry and technology. To date, several studies on marine species and most on terrestrial creatures have examined the production of enzymes (Balan *et al*., 2012). In this study, a few halophilic bacteria have been isolated, characterized, and identified, which colonized the rhizosphere of plants found along the coastal regions of Dakshina Kannada, India (Paramashree and Siddaraju, 2023). The identified halophilic bacteria of our study were characterized for their optimum physiological and biochemical parameters for maximum growth and enzymes production.

2.0 LITERATURE REVIEW

There are immense research on halophilic bacteria from different hypersaline environment involving the study of their cellular modifications, metabolisms, enzyme production and activities. The research on the broad spectrum of halophilic bacteria producing hydrolytic enzymes in varied ecological environments are few. The preliminary step in the breakdown of complex organic compound is hydrolysis, which in saline habitat has a significant impact on the metabolic cycle of nutrients. In this perspective, the research on halophilic bacteria in the field of environmental studies holds considerable significance (Moreno *et al*., 2013). In the recent studies, researchers screen for enzymes produced by halophilic bacteria by direct plating on the agar plates and there are just a few of environmental research (Moreno *et al*., 2013).

2.1 Global research on halophilic bacteria

The halophilic bacteria from saline lake, Algeria belonged to the genera *Bacillaceae*, *Planococcaceae* and *Staphylococcacea* of family Firmicutes and genera *Halomonadaceae* and *Idiomarinaceae* of family γ-Proteobacteria. The enzymatic activity was more in Firmicutes isolates than γ-Proteobacteria isolates. The isolates were inhibiting metal up to 5 mM concertation, highest minimum inhibitory concentration (MIC) was reported against copper and nickel. Higher level of metal tolerance was in *Bacillus*, *Halobacillus*, *Virgibacills* and *Halomonas*. 10 isolates showed antifungal activity (Menasria *et al*., 2019).

Mukhtar *et al*. (2019) have isolated halophilic bacteria from, Khewra Salt Mine, Pakistan. The isolates belonged to *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria* genera and had protease, lipase, cellulase, amylase, urease, gelatinase and catalase activity. The Shannon diversity index for isolates was 2.95. The optimum pH and temperature for both growth and activity of enzymes was pH 8 and 37 °C.

Fariq and Yasmin (2020) isolated, Halophilic bacteria from Khewra Salt Mines in Pakistan, were reported to produce biosurfactants. These biosurfactant had antibacterial and antifungal activities against *Fusarium solani, Aspergillus fumigatus, E. coli, Bacillus subtilis, Staphylococcus aureus, Aspergillus flavus, Enterococcus faecalis, Klebsiella pneumoniae*, and *Aspergillus niger*.

Yan *et al*. (2023) have isolated *Virgibacillus massiliensis* C, a halophilic bacterium from Qingdao, China's Yinjiashan Saltworks. This study employed halophilic bacteria, *Virgibacillus massiliensis* C, at both free and immobilized state, had carbonic anhydrase activity to extract Ca^{2+} ions from water at varying concentrations of NaCl. For the six days of incubation, the free

and immobilized bacteria generated 87% - 98%, of calcium ion degradation respectively. The bacteria's release of polysaccharide, protein, DNA, and compounds like humic acid aided in the elimination of Ca^{2+} ions. Because the immobilized bacteria could be regenerated and precultured, the industry would save money and remove Ca^{2+} ions more effectively.

Jafari *et al*. (2018) have isolated halophilic bacteria from the Howze-Soltan Playa in Iran's salty soil. The isolate reported to have antifungal activity against *A. parasiticus* NRRL 2999. The aflatoxins B1 and aflatoxin G1 production were inhibited by bacterial strains from the range of 15.1 - 98.9% and 57 is 0 - 99.6%. The highest and least inhibitory activity of Aflatoxin G1 was reported by bacterial strains *Staphylococcus xylosus* strain JCM 2418 and *Bacillus atrophaeus* strain JCM 9070. The bacterial isolates *B. aerius strain* 24 K and *P. dendritiformis* strain T168 inhibited 99.6% of aflatoxins B1 and that of *Paenibacillus apiarius strain* DSM 5581 was 57% of inhibition.

2.2 Indian research on halophilic microorganisms

The actinomycetes *Kocuria* sp. strain rsk4 from Porbandar, Gujarat, India, produced wide-ranging antimicrobial substance which has inhibitory activity against *Staphylococcus aureus* with lowest minimum inhibitory activity recorded was 30 μg/mL. The isolate showed growth well at 5 to 10% NaCl (w/v) and optimal production of antibiotic was at 5% NaCl, pH 5 - 9 and temperature 30 °C (Kumar and Jadeja, 2018).

Kumar *et al*. (2012) identified 108 bacteria from many saline areas of coastal regions of India. The bacteria belonging to *Halobacillus, Staphylococcus, Marinobacter, Bacillus, Virgibacillus, Oceanobacillus, Geomicrobium* and *Chromohalobacter* genera, had hydrolase enzymes i.e., amylase, protease and lipase. All isolates were found to be moderately halophilic which were needed salt in the range of 3 - 20% and optimum salt required was 5%. They identified that there is significant diversity in the halophiles depending upon the ecologies of saline habitats of India. The hydrolase produced were identified to be polyextremophile. The optimum growth was observed at pH range of 7.6 - 7.8, temperature range of 30.6 °C - 35.0 °C. *Marinobacter* sp. EMB8 produced maximum amylase, *Marinobacter* sp. EMB5 produced maximum lipase and *Bacillus sp.* EMB9 produced maximum protease respectively.

Meena *et al*. (2019) identified *Actinobacteria* of soil sediment of deep sea volcanic Barren Island, Andaman and Nicobar. The isolate belonged to genera *Stretomyces, Brevibacterium, Salinospora, Nocardiopsis, Nocardia, Saccaropolyspora, Saccaromonospora, Blastococcus, Rhodococcus* and *Georginia* had antibacterial property. The large range of antibacterial property was seen in *Streptomyces* sp. It was also reported that the isolates produced bioactive compounds.

The bacterial isolates from Arabian sea coasts, Dandi, Navsari, Gujarat, India and coastal districts of Junagadh and Porbandar in Saurashtra, Gujarat showed plant growth promoting properties (Kapadia *et al*., 2022; Reang *et al*., 2022).

Salgaonkar and Rodrigues (2019) have isolated *Halococcus* and *Halorubrum* from iodized crystal salt from, saltpan in Verdaranyam of Tamil Nadu, India.

Neelam *et al*. (2019) isolated *Piscibacillus* sp. strain C12A1 from Sambhar Lake, Jaipur, Rajasthan. When tested on MDA-MB-231 breast cancer cells, the isolate exhibited anticancer activity. The best growth conditions for the bacteria were 37 °C temperature, 10 to 15% NaCl (w/v) and pH 8. Growth was observed at 15 °C - 55 °C, 0 - 30% NaCl, pH 5 – 12.

Seven halophilic bacteria were identified by Subramanian and Gurunathan (2019) from the Kumta Coast, Marakkanam saltern in South India and Pichavaram mangrove forest. The *Bacillus* VITPS5, *Bacillus* VITPS12, *Bacillus* VITPS7, *Bacillus* VITPS16, *Bacillus* VITPS3, *Bacillus* VITPS14, and *Planococcus maritimus* VITP21 were the isolates that were identified. The isolate *Bacillus* VITPS3 had 3.18 times more metabolite when there was a 10% (w/v) NaCl solution present.

Yadav *et al*. (2021) have isolated halophilic bacteria from different regions of Tamil Nadu and Rajasthan. The isolates produced cellulase, lipase, and amylase and can tolerate salinity in the range of 10 - 25%.

2.3 Research on halophilic microorganisms in Karnataka, India

Nagaraju and Gundappagol (2020) have isolated halophilic bacteria from areas with high salinity of Raichur and Koppal districts, Karnataka. The *Bacillus albus*, *B. safensis*, *Staphylococcus xylosus*, *Bacillus cereus* etc., were identified isolates. The isolates reported to solubilize insoluble $ZnZO_3$, ZnO, Mica-tri-calcium phosphate at 3 - 10% NaCl (w/v).

Divakar *et al*. (2018) identified halophilic bacteria from sediment, soil, and water samples of mangrove habitats of Honavar, Kumta and Karwar in the Uttar Kannada district of Karnataka. It was discovered that *Bacillus pumilus* (accession no. MF472596) was tolerant to four harmful heavy metal ions, up to 1000 ppm of each: Cd^{2+}, Cu^{2+}, Fe^{3+}, and Ba^{2+}. Also, at neutral pH, the isolate could withstand up to 900 ppm of lead and eliminate almost 96% of the lead (Sahoo and Goli, 2020).

Jayachandra and Sulochana (2020) have isolated *Halobacillus* sp. strain JS6, from an arable soil sample of west coast in Karnataka, India. Even at pH 10 and 20% NaCl, the isolate showed growth at roughly 45 °C. The strain was discovered to be resistant to neomycin, colistin, cefotaxime, cefepime, and ceftazidime, according to an antibiogram. The isolate responded well to ciprofloxacin, ampicillin, and chloramphenicol.

Fifty halophilic bacteria have been isolated by Nagaraju *et al*. (2021) from salty soils of Raichur in Karnataka and Ganagavathi in Karnataka. The bacteria were positive for ACC deaminase test and promoted growth of chickpea plants under salt stress. Plants treated with *Bacillus safensis* (B. *safensis*) exhibited increased flowering pod production, branching.

Nagaraju *et al*. (2021) identified halophilic bacteria from the soils in the Karnataka towns of Raichur and Gangavathi. *Pseudomonas stutzeri* strain MN1 (HB-13), *Bacillus safensis* strain Lewis-Bac-3 (HB-5), and *Staphylococcus xylosus* strain C5 (HB-18) were reported to have plant growth promoting property in black gram plant in the presence of salt. The isolates showed growth up to 10 - 13% NaCl (w/v) and able to solubilize minerals such as, $ZnCO_3$, ZnO, Mica and tri-calcium phosphate.

Dhanya and Chandra (2021) have isolated bacteria such as, *Pseudoalteromonas* sp.YU16-DR3A, *Pseudoalteromonas* sp.YU16-DR6A, *Burkholderia* sp.YU16-RN3 and *Enterobacter* sp.YU16-RN5 from the coastal regions of Mangalore, Someshwara beach, Ullal (12˚47' 10.3524"N74˚51' 12.4344" E). The samples were taken from surface sea water, rock scrapings from offshore, coastal plant (*Derris elliptica*), and algae. The isolates *Burkholderia* sp.YU16-RN3 and *Enterobacter* sp.YU16-RN5 were reported to produce exopolysaccharides.

Ravi Teja and Chandra (2021) have isolated halophilic bacteria and fungi from the gut, gonad, coelomic fluid of *Stomopneustes variolaris* from St. Mary's Island Udupi District in the state of Karnataka. The bacteria were identified as *Vibrio neocaledonicus* strain LZ4 (B/Go4), *Vibrio alginolyticus* strain G16 (B/CGGo1), *Vibrio parahaemolyticus* strain 3KC1 (B/C3) and *Vibrio* sp. strain SOVt012 (B/C2) and fungi were identified as *Cladosporium cladosporioides* strain SCS10, *Cladosporium oxysporum* strain CBR23 (F/Go2), *Cladosporium colombiae* strain DFFSCS017 (F/G5) and *Corynespora cassiicola* strain ABS48 (F/Go3). All the four identified fungi and *Vibrio Neocaledonicus* strain LZ4 (B/Go4), were reported to produce amylase enzyme at pH 6 - pH 8 and temperature at 10 °C – 50 °C.

To our knowledge, there are no studies on halophilic bacteria in entire Arabian coastal regions of Dakshina Kannada (district.) of Karnataka state, India and especially there are no studies on rhizospheric halophilic bacteria of the Arabian coastal regions of Dakshina Kannada district, Karnataka, India. Hence to document and study the rhizospheric halophilic bacteria of the Arabian coastal regions of Dakshina Kannada we have structured our study into following objectives.

Objectives of the study:

1. Isolation and characterization of bacteria from the rhizosphere in coastal areas.

2. Screening and optimization of culture conditions for the beneficial microbes identified.

3. Screening and preliminary characterization of the halophilic enzymes.

4. Purification and characterization of potential industrially important enzymes from selected microbes.

3.0 MATERIALS AND METHODS

All chemicals and solvents were acquired from commercial suppliers of Sigma-Aldrich India, HiMedia India, ThermoFisher Scientific, Nice Chemicals Pvt. Ltd. and Loba Chemie Pvt. Ltd. and used as such without further purification.

3.1 Isolation and characterization of bacteria from the rhizosphere in coastal areas

3.1.1 Study region

The region selected for this study was, the Arabian coast of Dakshina Kannada district, of Karnataka state, in India. Dakshina Kannada's Arabian Sea shoreline stretches for about 43 km, from Mulki to Thalappady according to Gazetteers, Government of Karnataka. The sample collection region was the sea shore stretch from Sasihithlu beach in the north to Someshwara beach in the south (~36 km). The sample collection was performed based on stratified random sampling method (Singh and Mangat, 1996; Asiegbu *et al*., 2020; Paramashree and Siddaraju, 2023). The 36 km selected seashore stretch was evenly split into 6 km strata, and six sample collecting sites were chosen. such as, "Someshwara beach (N 12° 47' 11.58", E 74° 51' 12.35"), Ullal delta (N 12° 50' 8.52", E 74° 49' 57.36"), Lion's beach park (N 12° 53' 58.2", E 74° 48' 44.64"), Hosabettu beach (N 12° 58' 22.8", E 74° 47' 50.64"), NITK beach (N 13° 0' 51.12", E 74° 47' 17.16"), Sasihithlu beach (N 13° 4' 14.52", E 74° 46' 36.12")" (Paramashree and Siddaraju, 2023). The plants which were growing within 300 m away from the sea and which were also examined and discovered to be regularly developing in each of the six sample collection sites (Paramashree and Siddaraju, 2023). In all six sample collection sites (strata), eight common plants were identified, including *Casuarina equisetifolia, Cocos nucifera, Ixora coccinea, Thespesia populnea, Rhizophora mangle, Ipomoea purpurea* and *Opuntia*. There were only two locations where *Scaevola plumiere* was discovered: Sasihithlu Beach and NITK Beach. From each sample collection site, rhizospheric soil of all these mentioned plants were collected (Paramashree and Siddaraju, 2023).

3.1.2 Sample collection

Samples of the soil in the rhizosphere from the plants that were selected were taken in October and November of 2019. The sample collection sites' pH and temperature were recorded. Using a sterilized shovel, the plants were dug up, and the extra dirt was carefully shaken from the roots. The tightly adhering rhizospheric soil was collected. The samples of rhizospheric soil

transported to the lab in a polythene bag in a cooler (Kumar *et al*., 2012; Sahay *et al*., 2011; Teng *et al*., 2019).

3.1.3 Measurement of salinity of the rhizospheric soil samples

Using a conductivity meter, the salt content of the soil samples was determined (Hardie and Doyle, 2012; Rhoades, 1993)

3.1.4 Isolation of halophilic bacteria

The nutrient agar media (Himedia) with pH 7 ± 0.2 was used. This media was aseptically enhanced with various NaCl (w/v) concentrations. The NaCl concentrations used were 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, and 35% (w/v) (Sahay *et al*., 2011). This media was autoclaved for 20 min at 121 °C and 15 psi of pressure. After the media was autoclaved, it was transferred into sterile petri dishes and left to solidify. The samples of soil underwent ten folded serial dilutions using sterilized distilled water and each diluent was inoculated into media. The spread plate method was used to inoculate the diluents, and subsequently incubated for two days at 37 ºC. To obtain the pure culture, individual colonies were chosen and sub-cultured several times (Jafari *et al*., 2018; Sahay *et al*., 2011).

3.1.5 Colony characteristics

Study was done on the motility, form, size, margin, elevation, opacity, and pigmentation of the colonies (Breakwell *et al*., 2007).

3.1.6 Biochemical characteristics

The isolates underwent a series of biochemical tests, all of which were conducted in triplets (Elevi *et al*., 2004; Azhar *et al*., 2014).

For the catalase test the thin smear of the 24 hrs old bacterial isolates was made on sterile glass slides and on that, 1 to 2 drops of 3% H_2O_2 (v/v) were added and tested for effervescence. The presence of amylase, gelatinase, and urease was tested by inoculating the bacterial isolates onto media containing starch, gelatine, and urea, respectively, as a substrate and incubated for 24 hrs at 37 °C and assessed the results. For the oxidase test, the 24 hrs old bacterial colonies were picked using a sterile glass rod and smeared onto the oxidase disc (HiMedia) and observed for the development of dark blue or purple coloration within 30 sec. The indole test was performed by inoculating the culture tubes containing 5 mL tryptophan media with bacterial isolates and incubating for 24 hrs at 37 °C. The methyl red and VP tests were performed using an MR-VP

broth medium. The isolates were inoculated and incubated in MR-VP broth medium for 24 hrs at 37 °C and results were checked using methyl red indicator for methyl red test and VP reagent for VP test. For the carbohydrate fermentation test, the 20 mL screw-cap tubes were filled with 6 - 8 mL of carbohydrate broth, and the Durham tubes were inserted in an inverted position inside the screw cap tube. The color of the media was light red, and the pH was 7.4 ± 0.2. The screw cap tubes containing media were autoclaved at 121 °C for 15 min for sterilization. The screw cap tubes were aseptically inoculated with bacterial isolates and incubated at 37 °C for 24 - 48 hrs and observed for acid and gas production (Elevi *et al*., 2004; Azhar *et al*., 2014).

3.1.7 DNA isolation

The purified bacterial pelleted cells were obtained by centrifuging 1.5 mL of culture broth at 900 g for 5 min. The obtained pellet was resuspended in 0.5 mL SET buffer (75 mM NaCl, 25 mM EDTA, and 20 mM Tris) with 10 µL of lysozyme (10 mg/ml), and then the tubes containing the sample were incubated at 65 °C for 5 min in a water bath (Sahay *et al*., 2011). Different proportions of organic solvents were used in three steps for the isolation of genomic DNA. A mixture of chloroform: isoamyl alcohol (24:1) in equal volume to the sample was added, vortexed 3-5 times, and then centrifuged at 9000 g for 5 min. The aqueous phase was separated, and an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and centrifuged at 9000 g for 5 min. The aqueous phase was resuspended with an equal amount of chloroform: isoamyl alcohol (24:1) and centrifuged at 9000 g for 5 min. An equal amount of Propan-2-OL (isopropyl alcohol) was added to the upper aqueous phase. Then, the sample was mixed gently by inversion at room temperature and centrifuged at 9000 g for 3 minutes. The supernatant was separated, and the pelleted DNA was dissolved in 70 µL of NaCl 1.2 M (w/v) solution. 300 µL absolute cold ethanol was added to the DNA obtained, and aliquots were incubated at -20 °C for 7 min. The tubes containing DNA were centrifuged at 9000 g for 5 min, and the ethanol was discarded. The pellet was washed once with 70% cold ethanol and centrifuged at 9000 g for 5 min. The pellet was then dissolved in 50 µL deionized water by gentle vortexing and stored at -80 °C until further use (Paramashree and Siddaraju, 2023; Sahay *et al*., 2011; Stirling, 2003).

3.1.8 The PCR amplification of 16S rRNA gene

Amplification of the PCR was carried out at Department of Microbiology, College of Fisheries, Mangalore, Karnataka, India. The Bio-Rad thermal cycler was used to conduct the PCR reactions. The 16S rRNA gene was amplified using primers 27F

(5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5' TACGGTTACCTTGTTACGACTT 3'). There were 30 µL total in the PCR reaction mixture. 3 µL of 10x polymerase reaction buffer, 2 µL of DNA and 0.6 µL of dNTPs, 1 µL each of the primers 27F and 1492R, and Taq polymerase - 0.3 µL were all included in the reaction mixture (Paramashree and Siddaraju, 2023). The PCR amplification cycle consisted of the following steps: a 5 min initial denaturation at 94 °C, 40 cycles of 30 s at 94 °C, 40 s at 50 °C, 90 s at 72 °C, and a final 7 min extension period at 72 °C (Sahay *et al*., 2011; Frank *et al*., 2008; Patel *et al*., 2017; Ballav *et al*., 2015).

3.1.9 Gel electrophoresis of the PCR product

Electrophoresis was done for the PCR products using 1.2% (w/v) agarose gel in 1X TAE buffer. The gel was visualized in the Bio-Rad Gel Documentation system, and digital images were documented (Sahay *et al*., 2011).

3.1.10 Cleaning up of the PCR product

The PCR-amplified 16S rRNA was purified using NucleoSpin (Gel and PCR clean-up).

3.1.11 Sequencing and phylogenetic analysis

The sequencing was performed at Bioserve biotechnologies (India) Pvt. Ltd. Hyderabad. The two strands were sequenced using primers 27F and 1492R for the forward and reverse reactions, respectively (Paramashree and Siddaraju, 2023). The nucleotide sequences were ran using an automated DNA sequencer, the 3130x1 Applied Biosystems ABI Prism, using fluorescent terminators and di-deoxy cycle sequencing.

The GenBank database was utilized to identify the isolates. Data were translated into PHYLIP format and the Clustal Omega tool was used with default parameters to do the multiple sequence alignment and comparative investigations. The neighbor-joining technique was used in the MEGA 7 program to create the phylogenetic tree. After submitting the sequences to GenBank and obtained accession numbers (Paramashree and Siddaraju, 2023; Sahay *et al*., 2011; Jafari *et al*., 2018).

3.2 Screening and optimization of culture conditions for the beneficial microbes identified.

The impact of range of temperature, pH, and NaCl percentage (w/v), carbon sources, nitrogen sources and starch rich flours were analyzed. From this study, the optimum physiological and biological conditions for bacterial growth was identified. 50 mM sodium acetate used at pH 5.0

- 6.0, for pH 7.0 - 8.0 Tris-HCL and for pH 9.0 - 12.0 glycine-NaOH was used. Temperatures between 20 and 50 °C were tested. NaCl concentration from 0 - 10% (w/v) was tested (Neelam *et al*., 2019; Gupta *et al*., 2015; Donio *et al*., 2013). The qualitative test was performed by checking bacterial growth on nutrient agar plates. The range of physiological conditions at which bacteria showed growth were selected for quantitative estimation by growth curve studies.

The quantitative tests were done by growing the isolates in nutrient broth (HiMedia) with 1% NaCl (w/v) and adjusting the concentration of culture broth to 2.0×10^6 CFU/mL and 100 µL was added to 30 mL of nutrient broth (HiMedia) and cultured at 37 °C for 24 hrs. For every 1 h interval, the absorbance was measured at 600 nm (Gupta *et al*., 2015; Donio *et al*., 2013; Neelam *et al*., 2019). For obtaining pH from 5.0 - 6.0, 50 mM sodium acetate; for 7.0 - 8.0, Tris-HCL; and for pH from 9.0 - 11.0 and 12 - 13 glycine-NaOH were used. Temperature ranges from 20 – 50 °C and NaCl concentrations from 0 - 30% (w/v) were tested (Gupta *et al*., 2015). Different carbon sources such as, glucose, lactose, maltose, potato starch and starch-rich flours such as rice, wheat, maize, and sorghum and different nitrogen sources such as peptone, gelatine, beef extract, yeast extract, casein, skim milk powder, ammonium chloride, and urea were tested. The carbon in 1% (w/v) and nitrogen sources at a concentration of 0.8% (w/v) were added to the nutrient media (Gupta *et al*., 2015; Donio *et al*., 2013; Neelam *et al*., 2019).

3.2.1 Growth pattern of bacterial isolates at optimum physiological conditions

The isolates' culture concentration was calibrated to 2.0×10^6 CFU/mL (stock). 1% nutrient broth media (w/v) (HiMedia) containing 2% NaCl (w/v) and pH 7 ± 0.2 was prepared. 100 µL of stock broth inoculated into nutrient broth (30 mL) and kept at 35 °C, 200 rpm for 24 hrs. For every 1 h interval, the absorbance was measured at 600 nm (Gupta *et al*., 2015; Donio *et al*., 2013; Neelam *et al*., 2019).

3.2.2 Antibiotic sensitivity test

Antibiotic susceptibility tests were performed on the isolates using 19 distinct antibiotic discs (HiMedia). The Mueller Hinton Agar (HiMedia) media pH 7 ± 0.2 was used. Antibiotic discs were aseptically implanted after being streaked with bacterial isolates., and cultured for 24 hrs at 35 °C (Paramashree and Siddaraju, 2023). The tests were carried out using the disc diffusion, or "Kirby-Bauer," method. Using a ruler, the zone of inhibition was calculated. The results obtained were studied under CLSI guidelines and the isolates resistant, intermediate and sensitivity characters were analyzed (Paramashree and Siddaraju, 2023; Patel e*t al.*, 2017).

3.3 Screening and preliminary characterization of the halophilic enzymes.

The production of caseinase, cellulase, and lipase according to (Cojoc *et al*., 2009) and of oxidase, gelatinase, urease, catalase, and amylase according to (Azhar *et al*., 2014) were qualitatively assessed in the isolates (Paramashree and Siddaraju, 2023).

3.3.1 The qualitative analysis of effect of physiological parameters on production of enzymes in bacterial isolates

The isolates were analyzed for impact of salinity, pH, and temperature on enzymes production in bacterial isolates. The qualitative production of enzymes was analyzed by varying the pH from 7 to 11, temperature from 30 to 45 °C and salinity from 0% to 8% NaCl (w/v) (Balan *et al*., 2012, Karan *et al*., 2011, Gupta *et al*., 2015).

3.3.2 Quantitative estimation of gelatinase activity

After adjusting the culture broth concentration to 2.0×10^6 CFU/mL (stock), 30 mL of nutritional gelatine broth (HiMedia) pH 7 ± 0.2, 2% NaCl (w/v) was mixed with 100 µL of the broth and cultured at 35 °C for 24 hrs. The same media without bacterial inoculation was used as control. 0.1 mL of the liquified gelatine broth sample (crude enzyme) was included into the reaction mixture. In the reaction mixture, there was 0.3 mL of (0.2%) gelatine in water (w/v) and 0.2 mL of 150 mM Tris-HCl (pH 7.5) with 12 mM $CaCl_2$. After 30 min of incubation at 30 °C, in order to stop the reaction, add 0.6 mL (0.1 N) of HCl to the reaction mixture. The mixture's release of amino acid, which is linked correlated with the amount of gelatinase (Balan *et al*., 2012). The unit of measurement for gelatinase activity is µmole of leucine equivalent per min/mL of culture filtrate (Hamza and Hassan, 2006).

Using the ninhydrin technique, the quantity of liberated amino acids was determined (Reagents essential: ninhydrin reagent (Nice Chemicals), 150 µg/mL of standard amino acid stock solution, 50% ethanol, distilled water.

2 µL of the material was mixed with 1 mL of ninhydrin in 998 µL of distilled water. The tubes were placed in a bath of boiling water and incubated for 15 min. After adding 5 mL of solvent, the mixture was left for 10 min at room temperature. The identical mixture without the gelatine was used as a blank. Absorbance at 570 nm was measured. (Balan *et al*., 2012; Joseph *et al*., 2018).

3.3.3 Identification of optimum physiological conditions for maximum gelatinase activity in bacterial isolates

3.3.4 Effect of physiological parameters on gelatinase production

The culture concentration of the isolates was adjusted to 2.0×10^6 CFU/mL. 1% nutrient gelatine broth medium (w/v) (HiMedia) with 2% NaCl (w/v) was prepared pH was adjusted to required range using Tris-HCL and glycine-NaOH 100 µL of culture broth added to 30 mL of nutrient gelatine broth (w/v) and incubated at 35 °C for 24 hrs. After incubation, the gelatinase production was quantitatively estimated. Same procedure was repeated for salinity and temperature by varying their concentration and incubation time respectively (Balan *et al*., 2012; Karan *et al*., 2011).

3.3.5 Location of gelatinase enzyme production

In order to ascertain the type of gelatinase generated (extracellular or intracellular), the isolates' culture concentration was calibrated to 2.0×10^6 CFU/mL (stock). 100 µL (stock) inoculated into nutrient gelatine broth (HiMedia) (30 mL), pH 7 ± 0.2, 2% NaCl (w/v) and cultured at 35 °C for 24 hrs. The same media without bacterial inoculation was used as control. After incubation, the liquified gelatine broth sample (crude enzyme) were centrifuged for 15 min at 5000 g. Extracellular gelatinase was estimated using the supernatant. After mixing the pellet with lysis buffer that contained PMSF (phenylmethylsulphonyl fluoride), the lysis was carried out in a sonicator for 30 sec at a frequency of 20 kHz. The gelatinase was estimated using the lysed extract as above-mentioned method (Kanemitsu *et al*., 2001; Joseph *et al*., 2018).

3.3.6 Quantitative estimation of catalase enzyme activity

After adjusting the culture broth concentration to 2.0×10^6 CFU/mL (stock), 100 µL was added to nutritional broth (HiMedia) (30 mL) pH 7 ± 0.2 and 2% NaCl (w/v) and incubated at, 35 °C for 24 hrs (Paramashree and Siddaraju, 2023). The same media without bacterial inoculation was used as control. A thermostat spectrophotometer (Equip-Tronics) was used and wavelength was set to A240 at 25 °C. The hydrogen peroxide 0.036% solution was prepared in a 50 mM potassium phosphate buffer at 25 °C (pH 7.0 ± 0.2) and kept in a light-sensitive bottle between 15 and 30 °C. 50 mM potassium phosphate buffer, was taken as blank. 2.90 mL of prepared A solution of hydrogen peroxide was added to the quartz cuvette and inserted in spectrophotometer. 0.10 mL supernatant of 14 hrs old bacterial broth (stock) was introduced into the cuvette and quickly combined by inversion. At A240 wavelength, the amount of time

needed for the absorbance to drop from 0.45 to 0.40 units was noted (Yumoto *et al*., 2000; Cendrin *et al*., 1994)

Using the following formula, enzyme activity was determined,

$$\text{Units/ ml enzyme} = \frac{(3.45)\,(df)}{(time)\,(0.1)}$$

Where,

df = dilution factor

3.45 = Degradation of 3.45 µM of H_2O_2 in a 3.0 mL reaction mixture resulted in a drop in A240 from 0.45 to 0.40.

0.1 = enzyme added to the cuvette in mL

time = minutes for the A240 to drop from 0.45 to 0.40 absorbance units

3.3.7 Identification of optimum culture conditions for maximum catalase activity in bacterial isolates

The effect of pH, temperature, salinity was studied using 1% nutrient broth (HiMedia). Testing was done on a pH range of 7 to 11, a range of 0 to 8% (w/v) salinity and 30 to 45 °C temperature. Carbon sources, nitrogen sources, and starch rich flours on catalase activity was tested using M9 medium by varying 0.5% (w/v) carbon and nitrogen sources. Potato starch, lactose, maltose, mannitol and glucose were the various carbon sources that were utilized. Tests were conducted on starch-rich flours, including sorghum, rice, maize and wheat. Various forms of nitrogen, including gelatin, ammonium chloride, yeast extract, peptone, urea and beef extract, were tested (Joseph *et al*., 2018, Zeng *et al*., 2010, and Balan *et al*., 2012).

The culture concentration of the isolates was adjusted to 2.0×10^6 CFU/mL. 1% nutrient broth medium (w/v) (HiMedia) with 2% NaCl (w/v) was prepared, pH was adjusted to required range using Tris-HCL and glycine-NaOH. 100 µL of culture broth added to 30 mL of nutrient broth (w/v) and incubated at 35 °C for 24 hrs. After incubation, the catalase production was quantitatively estimated. Same procedure was repeated for salinity and temperature by varying their concentration and incubation time respectively (Fu *et al*., 2014; Cendrin *et al*., 1994; Dinçler and Aydemir, 2001; Zeng *et al*., 2010). The same media without the introduction of

microbes was used as control. After incubation, the catalase activity was quantitatively estimated as mentioned above (Yumoto *et al*., 2000; Cendrin *et al*., 1994).

3.3.7.1 Effect of carbon and nitrogen sources on catalase activity

The culture concentration of the isolates was adjusted to 2.0×10^6 CFU/mL. Mineral salt broth medium (w/v) was prepared. pH 7 and salinity was adjusted 2%. The different carbon sources at 0.5% and nitrogen sources at 0.8% were added. 100 µL of culture broth added to 30 mL of Mineral salt broth (w/v) incubated at 35 °C for 24 hrs. After incubation, the catalase production was quantitatively estimated (Balan *et al*., 2012; Joseph *et al*., 2018).

3.3.7.2 Location of catalase enzyme production

The culture concentration of the isolates UCM2, UCM3, UCM8 and UCM9 was adjusted to 2.0×10^6 CFU/mL (stock). 100 µL (stock) inoculated into 1% nutrient broth (HiMedia) (30 mL), pH 7 ± 0.2, 2% NaCl (w/v) and incubated at 35 °C, 200 rpm for 12-14 hrs. 5 mL broth samples underwent a 5 min centrifugation at 5000 g for 15 min. Extracellular catalase was measured using the supernatant. The pellet was combined with lysis buffer that included PMSF (phenylmethylsulphonyl fluoride) and the lysis was performed in a sonicator at a frequency of 20 kHz for 30 sec (Kanemitsu *et al*., 2001). The lysed extract was used to estimate catalase qualitatively as mentioned above (Cendrin *et al*., 1994; Yumoto *et al*., 2000).

3.3.7.3 Effect of growth on catalase activity in the bacterial isolates

The catalase activity of the isolate in different growth phase was determined. The culture concentration of the isolates UCM2, UCM3, UCM8 and UCM9 was adjusted to 2.0×10^6 CFU/mL (stock). 100 µL of stock culture inoculated in to 1% nutrient broth (HiMedia) (30 mL) with pH 7 ± 0.2, 2% NaCl (w/v) and kept at 35 °C for 12-14 hrs. At every 1 h, the growth of bacteria was checked at A600 nm and simultaneously the catalase activity was also calculated using above mentioned method (Karan *et al*., 2011).

3.4 Purification and characterization of potential industrially important enzymes from selected microbes.

3.4.1 Protein estimation of the bacterial broth

The culture concentration of the isolates UCM2, UCM3, UCM8 and UCM9 was adjusted to 2.0×10^6 CFU/mL (stock). 100 µL (stock) inoculated into standardized nutrient broth (HiMedia) (30 mL) with pH 7 ± 0.2, 2% NaCl (w/v) and incubated for 12 - 14 hrs at 200 rpm and 35 °C.

As a control, the identical medium devoid of bacterial inoculation was employed. The broth samples were assayed for the protein estimation using Lawry's method. BSA (0.2 mg/mL) was used as standard and tube without sample used as control. The sample tubes and control tube were added with 1 mL of Lowry's reagent. Lowry's reagent is made by combining solutions A and B in a 50:1 ratio (Solution B: 0.5% copper sulfate solution in 1% freshly made sodium potassium tartrate solution; Solution A: 2% sodium carbonate in 0.1 N NaOH). Following the incubation period, 30 min were spent at room temperature with the addition of 100 µL of FC reagent (Loba Chemie). Moreover, absorbance at 750 nm was measured following the incubation (Redmile-Gordon *et al*., 2013).

3.4.2 Storage stability of crude catalase enzyme

The 4 isolates such as UCM2, UCM3, UCM8 and UCM9 were tested for stability of their catalytic activity in room temperature and at 4 °C for 8 days. After adjusting the isolates' culture concentration to 2.0 x 10^6 CFU/mL, 100 µL was added to 30 mL of standardized nutritional broth (pH 7 ± 0.2) with 2% NaCl (w/v), and the mixture was cultured for 12 to 14 hrs at 35 °C. The same media without the introduction of microbes was used as control. The obtained supernatant after centrifuging the broth culture for 15 min at 5,000 g. At 4 °C and room temperature, the supernatant was kept in 5 mL portions each and tested for catalase activity up to 8 days (Dinçler and Aydemir, 2001).

3.4.3 Effect of ammonium sulphate percentage on catalase protein precipitation

After the isolates' culture concentration was brought down to 2.0 x 10^6 CFU/mL, 100 µL was added to 30 mL of standardized nutritional broth (pH 7 ± 0.2, 2% NaCl (w/v), and the mixture was cultured for 12 to 14 hrs at 35 °C and 200 rpm. After centrifuging the broth culture for 15 min at 5,000 g, the supernatant was recovered. 5 mL each of the supernatant was saturated with 5%, 10%, 15%, 20%, 35% and 50% ammonium sulphate separately and kept overnight at 4 °C (Duman and Kaya, 2013). The samples underwent a 20 min centrifugation at 10,000 rpm. The supernatants and precipitates of the isolates were subjected to dialysis for two days. Both the supernatant and the precipitate were tested for protein and catalase activity (Yumoto *et al*., 2000; Cendrin *et al*., 1994). The protein estimation of precipitates was performed using Lowry's method (Redmile-Gordon *et al*., 2013).

3.4.4 Partial purification of catalase protein from *Bacillus cereus* strain UCM 8

The partial purification of catalase protein was performed at, School of Life sciences, University of Hyderabad. The isolate *Bacillus cereus* strain UCM8 was selected for partial purification of catalase protein since its highest production at extreme physiological conditions.

The isolate UCM8's culture concentration was adjusted to 2.0×10^6 CFU/mL. from which 100 µL inoculated into standardized nutrient broth (30 mL), pH 7 ± 0.2, 2% NaCl (w/v), and total of 180 mL was inoculated and incubated for 14 hrs at 200 rpm and 35 °C. As a control, same media devoid of bacterial inoculation were employed. For fifteen minutes, the broth samples were centrifuged at 5,000 g. Supernatant was applied for purification of catalase protein (Kanemitsu *et al*., 2001). The supernatant was also assayed for protein using Bradford's method. BSA (1mg/mL) was used as standard. The tubes without sample taken as control. The sample tubes and control tubes were added with 800 µL of Bradford reagent (HiMedia) and incubated for 10 min at dark. At 595 nm, the amount of absorption was measured following incubation.

3.4.4.1 Ammonium sulfate precipitation

A volume of 180 mL of crude extract was adjusted to 35% saturation with ammonium sulfate and incubated overnight at 4 °C. Samples subjected to centrifugation at 10,000 rpm or 20 min. This precipitate was again suspended in a small amount of phosphate buffer (0.1 M) pH 7 ± 0.2 (Joseph *et al*., 2018; Balan *et al*., 2012).

3.4.4.2 Dialysis

In minimum volume of 0.1 M phosphate buffer, the precipitate was dissolved and dialyzed for 2 days in same buffer with slight shaking in magnetic stirrer. The dialyzed product was analyzed for protein content and catalase activity as previously described (Balan *et al*., 2012; Joseph *et al*., 2018).

3.4.4.3 Membrane filtration of the purified sample

One Mili liter of the dialyzed sample was added to Amicon filter 10 kDa and centrifuged for 20 min at 10,000 rpm. The sample was separated as supernatant and effluent. The supernatant and effluent both were tested for protein content and catalase activity (Balan *et al*., 2012; Joseph *et al*., 2018).

3.4.5 Native-polyacrylamide gel electrophoresis

The partially purified catalase protein was subjected to PAGE. acrylamide gel 10% (w/v) was used. The 10% separating Gel (10 mL): 2.5 mL of 40% acrylamide and bis acrylamide, 2.5 mL of 4X separating gel buffer, 5 mL distilled water, 50 µL of 10% Ammonium per sulphate solution (freshly prepared), 10 µL of TEMED (Loba Chemie). 40% acrylamide and bis acrylamide: Acrylamide -100 g, Bisacrylamide - 2.65 g, raise the volume to 250 mL using deionized water and kept in brown colored bottle at 4 °C. 4X separating gel buffer: 36.3 g in 150 mL of deionized water, pH 8.8 was adjusted with HCl and volume made up to 200 mL using distilled water. 10% APS: 1g of Ammonium per sulphate in 10 mL of deionized water and stored at 4 °C (each time prepared fresh). Stacking gel 4 mL: acrylamide bis-acrylamide solution (40%) - 0.4 mL, 4X staking gel buffer - 1 mL, deionized water - 2.6 mL, TEMED - 5 µL10% and APS - 20 µL. 4X stacking gel buffer: Tris - 15.1 g, deionized water - 40 mL, HCl was used to bring the pH down to 6.8, and 50 mL was the final volume. Electrophoresis buffer: 0.025 M Tris, 2 liter of distilled water, 0.192 M Glycine and pH 8.3. Pre-stained molecular weight standard such as Lactoglobulin (18.4 kDa), Trypsin Soyabean Inhibitor (20.1 kDa), Ovalbumin (43 kDa), Catalase (240 kDa) and BSA (67 kDa) for Native-PAGE (HiMedia) were used.

The purified sample was mixed with sample loading buffer (β-mercaptoethanol, bromophemol blue and glycerol). The gel was run for 30 min using vertical electrophoresis unit (BioRad). The native gels were run for 60 min at 4 °C and 150 V, or until the dye front touched the gel's bottom. The gel was stained for 30 min using Coomassie brilliant blue stain (50 mL of methanol, 40 mL of distilled water 10 mL of glacial acetic acid, and 0.1 g of CBB R250). The gel was destained for 2 to 3 days using destaining solution (a 1:1:8 ratio of methanol, glacial acetic acid, and distilled water) (Dresler *et al*., 2011; Pollock *et al*., 2019).

3.4.6 SDS PAGE gel electrophoresis of the purified sample

The partially purified catalase protein was subjected to SDS PAGE. 7.5% and 10% SDS gel was used. 30% acrylamide and bis acrylamide solution was used in the ratio 29.1: 0.8 in double distilled water and kept at 4 °C at dark. Resolving gel buffer pH 8.8: 60 mL of deionized water with 1.5 M Tris base. Deionized water was used to bring the volume up to 100 mL and HCl was used to modify the pH to 8.8. Gel buffer stacking pH 6.8: 0.5 M tris base in 60 mL deionized water. HCl was used to modify the pH, and distilled water was used to adjust the volume to 100 mL. 10% SDS: 100 mL of double-distilled water containing 10 g SDS. maintained at room

temperature. 10% APS: Always prepared fresh. 100 mL of double-distilled water with 10 g of ammonium per sulphate, maintained at 4 °C. LSD dye (Laemmli sample loading dye) used as a sample loading buffer: 2 mL of distilled water, 0.25 mL of 0.5 M Tris base (pH 6.8), 1 mL of 50% glycerol, 0.4 mL of 10% SDS, 2 mL of 5% β-mercaptoethanol, and 0.02 mL of 1% bromophenol blue are needed to get the total volume to 50 mL with distilled water. MW 10-180 kDa prestained protein ladder (Thermo Scientific PageRuler) was employed. The gel was run for 30 min at 100 V using vertical electrophoresis unit (BioRad). The gel was stained for 30 min using Coomassie brilliant blue stain (10 mL of glacial acetic acid, 50 mL of methanol, 0.1 g of CBB R250, and 40 mL of distilled water). The gel was destained for 2 to 3 days using destaining solution (methanol, glacial acetic acid and distilled water in the ratio 1:1:8) (Dao *et al*., 2023; Laemmli, 1970; Buamard and Benjakul, 2015; Mad-Ali and Benjakul, 2019).

3.4.7 Determination of molecular weight of the purified protein using SDS PAGE

The R_f value was used to compute the partially purified protein molecular weight. 10% SDS gel was used. The R_f value of each band of MW standards and the purified protein were calculated using the formula,

$$R_f = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by dye front}}$$

The log MW against R_f value was shown on a standard curve. The outstanding dependability of molecular weight prediction is demonstrated by the strong linear relationship between the protein's molecular weight and migration distance (Bio-Rad laboratories; Al-Bagmi *et al*., 2019; Latif *et al*., 2013).

3.4.8 Ferguson plot

This plot is used for purified protein. The purified protein and protein ladder of MW 10-180 kDa (Thermo Scientific PageRuler) was analyzed in different concentrations of SDS PAGE gel i.e., 7.5%, 10%, 12.5% and 15%. The R_f value for sample along with each band of protein ladder, in all the test concentration was calculated. The log relative mobility of protein as a function of gel concentration was plotted. This plot used for the calculation of the pure protein's MW (Tomioka *et al*., 2023; Sharma and Guptasarma, 2017).

3.4.9 Lineweaver Burk Plot

The catalase activity was quantified using previously mentioned method, against various H_2O_2 concentration i.e., from 2.5 to 30 mM (Chen *et al*., 2015; Zeng *et al*., 2010; Dinçler and Aydemir, 2001). The pure catalase enzyme's 1/V against 1/S data were used to plot the Lineweaver Burk graph. The K_m and V_{max} of free enzyme was calculated using the formula;

$$1/V = (K_m/V_{max})\ (1/S) + 1/Vmax$$

3.5 Statistical analysis

The statistical package for the social sciences (SPSS) 22 version was used to examine all of the data. Turkeys test was used to calculate the differences between the test and control groups using a One-way analysis of variance (ANOVA) and Two-way analysis of variance (without differentiation). The statistical significance between the groups was determined by using $p < 0.05$ and reporting the data as mean ± standard deviation (SD).

4.0 RESULTS

4.1 Isolation and characterization of bacteria from the rhizosphere in coastal areas

4.1.1 Study region

The 36 km sample collection region was equally divided into six sample collecting sites apart 6 km. Each sample collection site was divided into 3 areas such as three hundred meters, two hundred meters and three hundred meters from the coast. In each site at 100 m, plants such as *Sporobolus pungens*, *Ipomoea purpurea* and *Rhizophora mangle* were found, at 200 m plants such as *Opuntia* and *Cocos nucifera* were found and at 300 m plants such as *Casuarina equisetifolia, Thespesia populnea* and *Ixora coccinea* were found. The plant *Scaevola plumieri* was found at 200 m only in two sites such as Sasihithlu beach and NITK beach.

4.1.2 Measurement of pH and temperature of sample collecting sites

The pH and temperature of each selected plant from sample collection site was measured and average values for each plant of all six sites was derived. The pH was in the range pH 7.3 – 8.33 and temperature was in the range 35 °C – 40 °C.

4.1.3 Measurement of salinity of the rhizospheric soil samples

The salinity of each selected plant from sample collection site was measured and average values for each plant was derived. The salinity of the collected samples was in the range 26 – 345 mS/m.

4.1.4 Isolation of halophilic bacteria

The rhizospheric soil sample of each plant from all the sites were aseptically mixed into a 100 mL autoclaved distilled water. The nine sample solutions were obtained for all the nine plants. The solutions were mixed well and subjected to serial dilution as mentioned in materials and methods section. The serial diluted samples were plated on Nutrient medium supplemented with NaCl percentage from 1% to 8% (w/v) and obtained ninety different types of bacterial colonies. Out of ninety isolates, thirty-two prominently growing isolates were selected for further characterization.

4.1.5 Colony characteristics

The colonies were pigmented as yellow, white, peach, pink, red, cream, lemon yellow, orange, etc., and the surface of the colonies was smooth, rough, glistening. Form of the colonies were, circular, punctiform, rhizoid, irregular and the margin of the colonies were entire, filamentous, undulate, lobate.

4.1.6 Biochemical characteristics

The 32 bacterial isolates were given codes representing its origin: CE - *Casuarina equisetifolia*, CN - *Cocos nucifera*, IP - *Ipomoea purpurea*, IC - *Ixora coccinea*, Op – *Opuntia*, RM - *Rhizophora mangle*, ScP - *Scaevola plumiere*, SP - *Sporobolus pungens*, TP - *Thespesia populnea*. All isolates from each plant origin were given numerical numbers ex; TP1 and TP2 were two isolates from *Thespesia populnea*. We have found nine isolates such as IP5, IP7, CN1, RM1, IP2, RM6, RM4, RM3 and CN3 that produces catalase and/or gelatinase (Paramashree and Siddaraju, 2023). All the results of colony characteristics and biochemical characteristics were analysed using Burgey's Manual of determinative bacteriology for the purpose of identification (Bergey, 1994).

4.1.7 Phylogenetic analysis and isolate identification

The isolates such as, IP5, IP7, CN1, RM1, IP2, RM6, RM4, RM3 and CN3 were having catalase and gelatinase activity, hence were selected for further phylogenetic analysis. The isolated DNA of all the nine isolates were subjected to PCR amplification of 16S rRNA gene. Approximately 1250 bp of PCR product was recovered for each isolate. The DNA sequences of the nine isolates, obtained from the 3130x1 Applied Biosystems ABI prism automated DNA sequencer was converted into FASTA format using the software CHROMAS (Paramashree and Siddaraju, 2023).

The resulting sequences underwent BLAST analysis. The results of the BLAST analysis indicate that the isolates are members of the following genera: *Bacillus*, *Citrobacter*, *Rhizobium*, *Mesobacillus*, *Ensifer*, *Luteimonas*, and *Microvirga*. The bacterial isolates IP5 and IP7 had 97.56% and 99.56% similarity to *Luteimonas marina* strain PP-R1, *Ensifer* sp. AC01b respectively; CN1 and RM1 had 94.26% and 98.10% similarity to *Mesobacillus subterraneus* strain ROA039, *Cellulosimicrobium funkei* strain CB17 respectively; IP2 and RM6 had 98.67% and 93.87%, respectively, to *Rhizobium pusense* strain NAC59 and *Bacillus subtilis* strain BS16045. RM4 and RM3 had 84.34%, 98.88% similarity to *Citrobacter freundii* strain

UKMB13 and *Bacillus cereus* strain R25-19. CN3 had 98.97% similarity to *Microvirga* sp. strain CAU (Paramashree and Siddaraju, 2023).

For each isolate, the six highest similarity sequences were considered and their FASTA sequences were downloaded. The sequences were subjected to multiple sequence alignment using CLUSTAL ω. The sequences underwent to phylogenetic analysis and phylogenetic tree was constructed using the software MEGA 7. Phylogenetic tree showed closely related species. The branches display the bootstrap values. The nine isolates such as IP5, IP7, CN1, RM1, IP2, RM6, RM4, RM3 and CN3 were given strain codes such as UCM1, UCM2, UCM3, UCM4, UCM5, UCM6, UCM7, UCM8 and UCM9 respectively. UCM indicates University College Mangalore.

The nine isolates were identified as, *Luteimonas marina* strain UCM1, *Ensifer sojae* strain UCM2, *Mesobacillus subterraneus* strain UCM3, *Cellulosimicrobium funkei* strain UCM4, *Rhizobium pusense* strain UCM5, *Bacillus subtilis* strain UCM6, *Citrobacter freundii* strain UCM7, *Bacillus cereus* strain UCM8 and *Microvirga* sp. strain UCM9 (Table 1.0). The sequences were submitted to GenBank® and the accession numbers obtained (Table 1.0).

Table 1.0: The accession numbers of the all nine identified isolates

Sl. no.	Isolate code	Strain	Identified as	Accession numbers
1	IP5	UCM1	*Luteimonas marina* strain UCM1	MW883185
2	IP7	UCM2	*Ensifer sojae* strain UCM2	MW819715
3	CN1	UCM3	*Mesobacillus subterraneus* strain UCM3	MW819716
4	RM1	UCM4	*Cellulosimicrobium funkei* strain UCM4	MW883186
5	IP2	UCM5	*Rhizobium pusense* strain UCM5	MW819717
6	RM6	UCM6	*Bacillus subtilis* strain UCM6	MW883187
7	RM4	UCM7	*Citrobacter freundii* strain UCM7	MW883188
8	RM3	UCM8	*Bacillus cereus* strain UCM8	MW883189
9	CN3	UCM9	*Microvirga* sp. strain UCM9	MW819718

4.2 Screening and Optimization of culture conditions for the beneficial microbes identified.

4.2.1 Qualitative investigation of the impact of physiological circumstances on the development of bacterial isolates

The bacterial isolates such as *Luteimonas marina* strain UCM1 and *Microvirga* sp. strain UCM9 showed growth at NaCl % (w/v) range of 0 - 2% and temperature range 30 - 40 °C. The bacterial isolates such as, *Rhizobium pusense* strain UCM5*, Ensifer sojae* strain UCM2*,* and *Cellulosimicrobium funkei* strain UCM4 showed growth at NaCl % (w/v) range of 0 - 5% and temperature range 30 - 40 °C. The bacterial isolates such as *Citrobacter freundii* strain UCM7*, Mesobacillus subterraneus* strain UCM3, *Bacillus cereus* strain UCM8 *and Bacillus subtilis* strain UCM6 showed growth at NaCl % (w/v) range 0 - 8% and temperature range 30 - 45 °C. All the nine isolates, showed growth at pH range 7 - 11 (Paramashree and Siddaraju, 2023).

4.2.2 Quantitative analysis of effect of pH on bacteria growth

The growth pattern of all the isolates were within 24 hrs and from pH 7 to pH 11. *Cellulosimicrobium funkei* strain UCM4, *Rhizobium pusense* strain UCM5, *Mesobacillus subterraneus* strain UCM6, *Citrobacter freundii* strain UCM7, *Bacillus cereus* strain UCM8, *Microvirga sp.* strain UCM9 and *Mesobacillus subterraneus* strain UCM3 showed optimum growth at broad range of pH i.e., pH 7 and pH 8. Isolate *Luteimonas marina* strain UCM1 showed optimum growth at pH 8 and isolate *Ensifer sojae* strain UCM2 showed optimum growth at pH 7 (Paramashree and Siddaraju, 2023).

4.2.3 Quantitative analysis of effect of salinity on bacteria growth

The growth pattern of the isolates was within 24 hrs and NaCl % (w/v) from 0 - 8%. Isolate *Mesobacillus subterraneus* strain UCM3 showed optimum growth at 0%, 1% and 2% NaCl (w/v). *Luteimonas marina* strain UCM1, *Ensifer sojae* strain UCM2, *Cellulosimicrobium funkei* strain UCM4 showed optimum growth at 1% and 2% NaCl (w/v). *Rhizobium pusense* strain UCM5, *Bacillus subtilis* strain UCM6, *Citrobacter freundii* strain UCM7, *Bacillus cereus* strain UCM8 and *Microvirga* sp. strain UCM9 showed optimum growth at 2% NaCl (w/v) (Paramashree and Siddaraju, 2023).

4.2.4 Quantitative analysis of effect of temperature on bacteria growth

The isolates' pattern of growth was within 24 hrs and temperature range from 30 - 45 °C. Isolate *Rhizobium pusense* strain UCM5, *Bacillus subtilis* strain UCM6, *Citrobacter freundii* strain UCM7, *Bacillus cereus* strain UCM8 and *Microvirga* sp. strain UCM9 showed optimum growth at 35 °C. *Luteimonas marina* strain UCM1, *Ensifer sojae* strain UCM2, *Mesobacillus subterraneus* strain UCM3, *Cellulosimicrobium funkei* strain UCM4 showed optimum growth at temperature range 30 °C and 35 °C (Paramashree and Siddaraju, 2023).

4.2.5 Growth pattern of bacterial isolates at optimum physiological conditions

All the isolates showed optimum growth at pH 7 ± 0.2, 2% NaCl (w/v) and 35 °C temperature. All the isolates were studied for their growth pattern in at these physiological conditions.

4.2.6 Effect of carbon sources on growth of isolates

The isolated bacteria displayed growth in different carbon sources tested. The isolates, *Luteimonas marina* strain UCM1, *Mesobacillus subterraneus* strain UCM3, *Ensifer sojae* strain UCM2, *Cellulosimicrobium funkei* strain UCM4, and *Bacillus subtilis* strain UCM6 showed optimum growth in glucose as carbon source. The isolates, *Microvirga sp.* strain UCM9 and *Bacillus cereus* strain UCM8 showed optimum growth in glucose and maltose as carbon source. Isolate *Rhizobium pusense* strain UCM5 showed optimum growth in lactose as carbon source. Isolate *Citrobacter freundii* strain UCM7 showed optimum growth in potato starch as carbon source (Paramashree and Siddaraju, 2023).

4.2.7 Effect of nitrogen sources on growth of isolates

The isolates showed growth in different nitrogen sources. The isolates *Ensifer sojae* strain UCM2 and *Cellulosimicrobium funkei* strain UCM4 showed optimum growth in peptone, beef extract and gelatine as nitrogen source. Isolates *Mesobacillus subterraneus* strain UCM3 showed optimum growth in peptone, beef extract and yeast extract as nitrogen sources. *Bacillus subtilis* strain UCM6, *Luteimonas marina* strain UCM1 and *Bacillus cereus* strain UCM8 showed optimum growth in peptone as nitrogen source. *Rhizobium pusense* strain UCM5 showed optimum growth in gelatine as nitrogen source. *Citrobacter freundii* strain UCM7 showed optimum growth in beef extract as nitrogen source. *Microvirga* sp. strain UCM9 showed optimum growth in peptone and gelatine as nitrogen source.

4.2.8 Effect of starch rich flour sources on growth of isolates

The growth of the isolates in different starch rich flours were tested. Isolates *Bacillus cereus* strain UCM8, *Mesobacillus subterraneus* strain UCM3, *Bacillus subtilis* strain UCM6, *Cellulosimicrobium funkei* strain UCM4, *Rhizobium pusense* strain UCM5 and *Microvirga* sp. strain UCM9 showed optimum growth in wheat. *Luteimonas marina* strain UCM1 showed optimum growth in maize. *Ensifer sojae* strain UCM2 showed optimum growth in sorghum. *Citrobacter freundii* strain UCM7 showed optimum growth in sorghum and wheat (Paramashree and Siddaraju, 2023).

Table 2.0 Antibiotic sensitivity test result

Sl. No.	antibiotic	code	Conc (mg)	Resistant (R), Sensitivity (S), Intermediate (I) property of identified isolates								
				UCM1	UCM2	UCM3	UCM4	UCM5	UCM6	UCM7	UCM8	UCM9
1.	Chloramphenicol	C	30	R	S	S	I	I	S	R	R	I
2.	Azithromycin	AZM	30	R	S	S	R	S	S	R	I	R
3.	Ciprofloxacin	CIP	5	S	S	S	S	R	S	S	R	R
4.	Erythromycin	E	15	S	S	S	S	S	S	I	S	S
5.	Penicillin-G	P	2	I	S	S	I	I	S	I	I	S
6.	Ampicillin	AMP	10	I	S	S	S	I	S	I	I	S
7.	Cefazolin	CZ	30	I	S	S	R	I	S	I	I	S
8.	Tobramycin	TOB	30	S	S	S	I	S	S	S	S	S
9.	Clindamycin	CD	2	R	R	I	R	I	R	I	R	R
10.	Nalidixic acid	NA	S	R	I	S	I	I	I	I	R	S
11.	Cefadroxil	CFR	30	R	I	S	S	R	S	I	R	R
12.	Cephalothin	CEP	30	I	S	S	I	I	S	I	I	S
13.	Bacitracin	B	8	I	S	I	S	I	S	I	I	I
14.	Oxacillin	OX	5	I	I	S	I	I	S	I	I	I
15.	Ampicillin/Cloxacilin	AX	10	I	S	S	I	I	S	R	I	S
16.	Amoxyclav	AMC	10	I	S	S	R	I	S	I	I	S
17.	Gentamicin	GEN	50	S	S	S	S	S	S	S	S	S
18.	Vancomycin	VA	10	S	R	S	S	I	S	I	I	S
19.	Streptomycin	S	S	S	S	S	S	I	S	S	S	I

4.3 Screening and preliminary characterization of the halophilic enzymes.

4.3.1 Screening for enzyme production and qualitative investigation of the impact of physiological factors on enzyme production.

All nine isolates were positive for catalase. *Luteimonas marina* strain UCM1, *Rhizobium pusense* strain UCM5, *Cellulosimicrobium funkei* strain UCM4, *Microvirga* sp. strain UCM9 and *Bacillus cereus* strain UCM8 showed positive for gelatinase and oxidase (Paramashree and Siddaraju, 2023). *Luteimonas marina* strain UCM1, *Rhizobium pusense* strain UCM5, *Ensifer sojae* strain UCM2, *Citrobacter freundii* strain UCM7, *Mesobacillus subterraneus* strain

UCM3 were also positive for urease. And all the isolates were negative for remaining enzymes tested. The effect of physiological parameters on enzymes production is given in Table 3.0.

Table 3.0: Effect of physiological parameters on enzyme production

Sl. no	isolates	Enzymes produced	pH	Temp (°C)	NaCl (w/v)
1	*Luteimonas marina* strain UCM1	gelatinase, catalase, oxidase, urease	7 - 11	30 - 40	0 - 2
2	*Rhizobium pusense* strain UCM5	gelatinase, catalase, oxidase, urease	7 - 11	30 - 40	0 - 5
3	*Cellulosimicrobium funkei* strain UCM4	gelatinase, catalase, oxidase	7 - 11	30 - 40	0 - 5
4	*Bacillus cereus* strain UCM8	gelatinase, catalase, oxidase	7 - 11	30 - 45	0 - 8
5	*Microvirga sp*. strain UCM9	gelatinase, catalase, oxidase	7 - 11	30 - 40	0 - 2
6	*Ensifer sojae* strain UCM2	gelatinase, catalase, urease	7 - 11	30 - 40	0 - 5
7	*Citrobacter freundii* strain UCM7	catalase, oxidase, urease	7 - 11	30 - 45	0 - 8
8	*Mesobacillus subterraneus* strain UCM3	catalase, urease	7 - 11	30 - 45	0 - 8
9	*Bacillus subtilis* strain UCM6	catalase	7 - 11	30 - 45	0 - 8

GELATINASE

4.3.2 Quantitative estimation of gelatinase activity

Out of nine isolates six isolates such as *Luteimonas marina* strain UCM1, *Bacillus cereus* strain UCM8, *Ensifer sojae* strain UCM2*, Rhizobium pusense* strain UCM5*, Cellulosimicrobium funkei* strain UCM4*, and Microvirga sp.* strain UCM9 showed gelatinase activity (Paramashree and Siddaraju, 2023). The isolates showed gelatinase activity in the range from 26,611 U/mL to 57,222 U/mL (Figure 1.0).

4.3.3 Effect of pH on gelatinase activity

The gelatinase activity of the isolates was observed from pH 7 - 11. The gelatinase activity of the isolates found to be stable along the pH changes. The isolates, *Ensifer sojae* strain UCM2 and *Rhizobium pusense* strain UCM5 showed highest gelatinase activity at pH 7. The isolates, *Luteimonas marina* strain UCM1 and *Microvirga* sp. strain UCM9 showed maximum gelatinase activity at pH 8. *Cellulosimicrobium funkei* strain UCM4 showed maximum gelatinase activity at pH 10. *Bacillus cereus* strain UCM8 showed maximum gelatinase activity at pH 10 and pH 11.

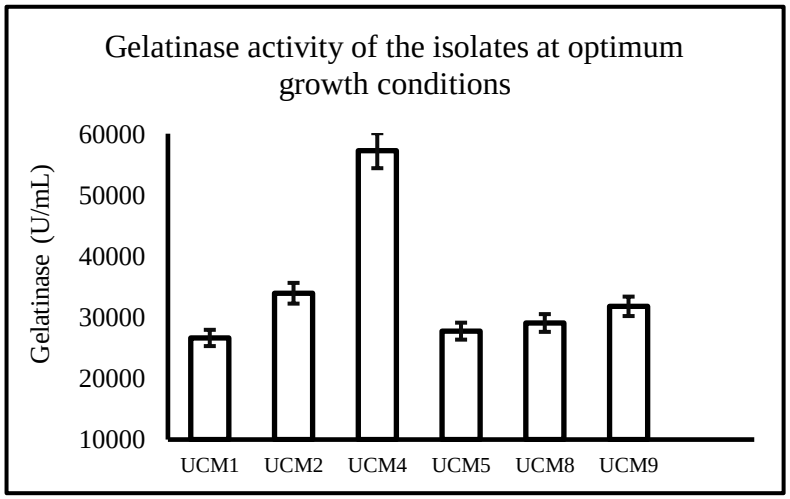

Figure 1.0: The gelatinase activity of the isolates at optimum growth conditions. The gelatinase activity of the isolates was determined at pH 7, 2% NaCl (w/v) and 35 °C. A mean ± SD (n = 3) was used to express the data.

4.3.4 Effect of salinity on gelatinase activity

All the six isolates showed gelatinase activity from 0 - 2% NaCl (w/v). The isolates, *Ensifer sojae* strain UCM2, *Rhizobium pusense* strain UCM5 and *Cellulosimicrobium funkei* strain UCM4 showed gelatinase activity up to 5% NaCl (w/v) and highest activity at 3% NaCl (w/v).

Isolates *Luteimonas marina* strain UCM1, *Microvirga* sp. strain UCM9 and *Bacillus cereus* strain UCM8 showed highest gelatinase activity at 2% NaCl (w/v). *Bacillus cereus* strain UCM8 showed gelatinase activity up to 8% NaCl (w/v).

4.3.5. Effect of temperature on gelatinase activity

All the six isolates showed gelatinase activity from 30 - 40 °C. *Bacillus cereus* strain UCM8 showed gelatinase activity up to 45 °C. Isolates *Luteimonas marina* strain UCM1, *Cellulosimicrobium funkei* strain UCM4 and *Microvirga* sp. strain UCM9 showed highest gelatinase activity at 30 °C. Isolates *Ensifer sojae* strain UCM2 and *Bacillus cereus* strain UCM8 showed highest gelatinase activity at 35 °C. Isolate *Rhizobium pusense* strain UCM5 showed highest gelatinase activity at 30 °C and 40 °C.

4.3.6 Location of gelatinase enzyme production in the bacterial isolates

All the six isolates showed extracellular gelatinase enzyme production.

CATALASE

4.3.7 Quantitative estimation of catalase

The catalase activity in *Luteimonas marina* strain UCM1 was 11,567 U/mL, in *Ensifer sojae* strain UCM2 was 34,167 U/mL, in *Mesobacillus subterraneus* strain UCM3 was 68,333 U/mL, in *Cellulosimicrobium funkei* strain UCM4 was 23,167 U/mL, in *Rhizobium pusense* strain UCM5 was 5,650 U/mL, in *Bacillus subtilis* strain UCM6 was 5,733 U/mL, in *Citrobacter freundii* strain UCM7 was 23,333 U/mL, in *Bacillus cereus* strain UCM8 was 29,714 U/mL and in *Microvirga* sp. strain UCM9 was 34,667 U/mL (Figure 2.0).

4.3.8 Effect of pH on catalase activity

All the nine isolates showed catalase activity from pH 7 to 11. All the isolates showed nearly same catalase activity from pH 7 to pH 9. The isolates, *Luteimonas marina* strain UCM1, *Mesobacillus subterraneus* strain UCM3 and *Citrobacter freundii* strain UCM7 showed maximum at pH 7. The isolates, *Ensifer sojae* strain UCM2, *Rhizobium pusense* strain UCM5 and *Bacillus subtilis* strain UCM6 showed maximum catalase activity at pH 9. Isolate, *Cellulosimicrobium funkei* strain UCM4 showed maximum catalase at pH 7 and pH 8. Isolates *Microvirga* sp. strain UCM9 and *Bacillus cereus* strain UCM8 showed maximum catalase activity at pH 8 and pH 10 respectively (Paramashree and Siddaraju, 2023).

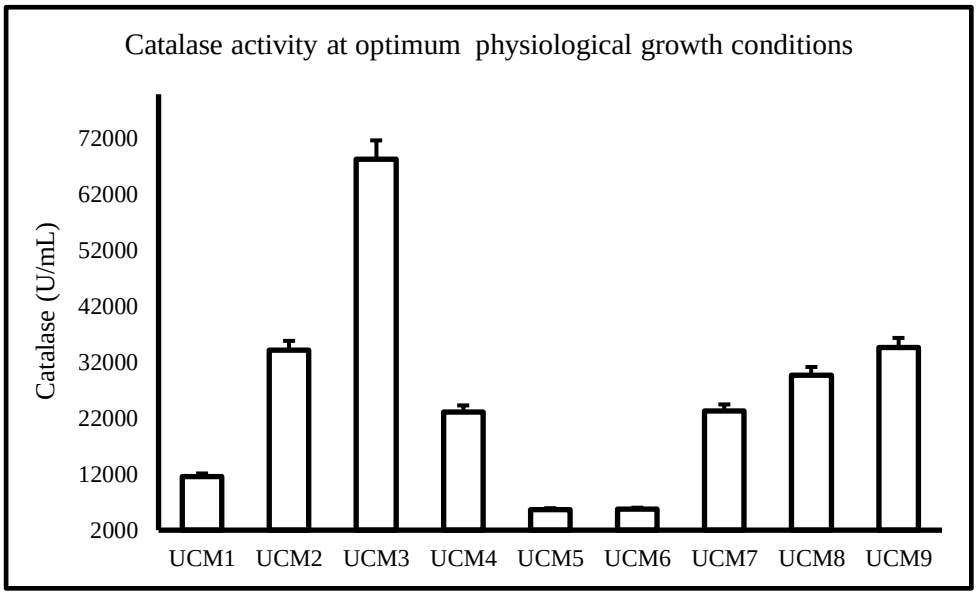

Figure 2.0: The catalase activity of the bacterial isolates. The catalase activity of the isolates was determined at pH 7, 2% NaCl (w/v) and 35 °C. A mean ± SD (n = 3) was used to express the data.

4.3.9 Effect of salinity on catalase activity

The isolates showed catalase activity from 0 - 8% NaCl (w/v). The isolates, *Mesobacillus subterraneus* strain UCM3*, Ensifer sojae* strain UCM2*,* and *Luteimonas marina* strain UCM1 and *Microvirga* sp. strain UCM9 showed maximum catalase activity at 2% NaCl (w/v). *Citrobacter freundii* strain UCM7*, Bacillus subtilis* strain UCM6*, Rhizobium pusense* strain UCM5*,* and *Cellulosimicrobium funkei* strain UCM4 showed maximum catalase activity at 3% NaCl (w/v) (Paramashree and Siddaraju, 2023). Isolate *Bacillus cereus* strain UCM8 showed maximum catalase activity at 5% NaCl (w/v). Isolates UCM4, UCM5, UCM7 and UCM8 showed similar catalase activity from 0 - 5% NaCl (w/v) (Paramashree and Siddaraju, 2023).

4.3.10 Effect of temperature on catalase activity

The isolates showed catalase activity from temperature 30 - 45 °C. All the isolates showed activity from 30 - 40 °C. Isolates, *Bacillus subtilis* strain UCM6*, Bacillus cereus* strain UCM8*, Mesobacillus subterraneus* strain UCM3*, Cellulosimicrobium funkei* strain UCM4*, Luteimonas marina* strain UCM1, *Ensifer sojae* strain UCM2, *Rhizobium pusense* strain UCM5 and *Citrobacter freundii* strain UCM7 showed maximum catalase activity at 30 °C (Paramashree and Siddaraju, 2023). Isolate *Microvirga* sp. strain UCM9 showed maximum catalase activity at 35 °C (Paramashree and Siddaraju, 2023).

4.3.11 Effect of carbon and nitrogen sources on catalase activity of the isolates.

The isolates which were producing maximum catalase activity were selected for further characterization. The isolates selected were, *Ensifer sojae* strain UCM2, *Mesobacillus subterraneus* strain UCM3, *Microvirga* sp. strain UCM9 and *Bacillus cereus* strain UCM8 (Paramashree and Siddaraju, 2023). These four isolates showed different catalase activity in different carbon sources tested. Isolates, *Ensifer sojae* strain UCM2, *Mesobacillus subterraneus* strain UCM3 and *Bacillus cereus* strain UCM8 showed maximum catalase activity in wheat as carbon source. The isolate *Microvirga* sp. strain UCM9 showed maximum catalase activity in lactose as carbon source. The isolates were showing different catalase activity in different nitrogen sources tested. The isolates such as, *Ensifer sojae* strain UCM2, *Microvirga* sp. strain UCM9 and *Bacillus cereus* strain UCM8 showed maximum catalase activity in gelatine as nitrogen source. *Mesobacillus subterraneus* strain UCM3 showed maximum catalase activity in beef extract as nitrogen source.

4.3.12 Location of catalase enzyme production in bacterial isolates.

The isolates, *Ensifer sojae* strain UCM2, *Microvirga* sp. strain UCM9 and *Bacillus cereus* strain UCM8 showed extracellular catalase production and *Mesobacillus subterraneus* strain UCM3 showed both extracellular and intracellular catalase production (Paramashree and Siddaraju, 2023).

4.3.13 Effect of growth phase of the bacteria on catalase activity

The catalase activity in all the four isolates increase along the exponential phase of the bacterial growth and the maximum catalase activity was seen at 12 - 18 hrs of incubation time.

4.4 Purification and characterization of potential industrially important enzymes from selected microbes.

4.4.1 Estimation of protein in the culture broth of the isolates.

The standard curve was plotted for Lowry's method. The protein content in the broth sample of isolate, *Ensifer sojae* strain UCM2 was 1.35 mg/mL, *Mesobacillus subterraneus* strain UCM3 was 1.29 mg/mL, *Bacillus cereus* strain UCM8 was 1.45 mg/mL and *Microvirga* sp. strain UCM9 was 1.65 mg/mL.

4.4.2 Storage stability of crude catalase enzyme

All the four isolates showed slight increase in catalase activity up to 40 hrs of incubation at room temperature and later the activity kept on gradually decreasing up to 180 hrs. The isolates, *Ensifer sojae* strain UCM2, *Mesobacillus subterraneus* strain UCM3 and *Microvirga* sp. strain UCM9 showed gradual decrease in catalase activity with the increase of incubation time at 4 °C. The catalase activity of *Bacillus cereus* strain UCM8 at 4 °C incubation, gradually decreases up to 80 hrs of incubation and completely loses the activity beyond 80 hrs.

4.4.3 Effect of ammonium sulphate percentage on catalase protein precipitation

The isolates *Ensifer sojae* strain UCM2 and *Bacillus cereus* strain UCM8 showed maximum catalase protein precipitation at 35% ammonium sulphate concentration. The isolates, *Mesobacillus subterraneus* strain UCM3 and *Microvirga* sp. strain UCM9 showed maximum catalase protein precipitation at 50% ammonium sulphate.

4.4.4 Partial purification of catalase protein from *Bacillus cereus* strain UCM 8

The isolate *Bacillus cereus* strain UCM8 showed maximum catalase activity at extreme physiological condition compared to other three strains. The *Bacillus cereus* strain UCM8 showed activity from pH 7 - 11, maximum activity at pH 10, 0 - 8% NaCl (w/v) maximum activity at 5% NaCl (w/v) and 30 - 45 °C and retain ~47% of its original activity at 45 °C. Hence, it was selected for further purification studies.

4.4.4.1 Membrane filtration of the purified sample

The dialyzed sample of the isolate *Bacillus cereus* strain UCM8 showed 0.82 mg/mL protein and 22,458 U/mL catalase activity. The membrane filtration (AMICON 10kDa) resulted in supernatant and effluent. The protein content and catalase activity of the supernatant was 0.7 mg/mL and 23,574 U/mL respectively. The protein content of the effluent was 0.30 mg/mL and had no catalase activity.

4.4.4.2 Polyacrylamide gel electrophoresis

The native PAGE resulted in thin bands of proteins and catalase protein was found to be ~50 KDa molecular size. The SDS PAGE of the partially purified catalase protein sample showed that the molecular size of catalase protein was ~50 KDa

4.4.5 Determination of the purified protein's molecular weight via SDS PAGE

As mentioned in section 3.4.7 of materials and method chapter, the molecular weight was determined. The molecular weight was ~49 KDa.

4.4.6 Ferguson plot

As mentioned in section 3.4.8 of materials and method chapter, the molecular weight was determined. The molecular weight according to Ferguson plot was ~56 KDa.

4.4.7 Lineweaver Burk Plot

The Lineweaver Burk Plot was given values of, Km = 0.022 and Vmax = 0.58.

5.0 DISCUSSION

To survive in hostile environments, bacteria and other microorganisms produce extremotolerant enzymes and secondary metabolites. The extremophilic bacteria that produce various enzymes and proteins have been the subject of much more research in recent years. The enzymes from these extremophilic bacteria are very important in a variety of industries, including food processing, biomedicine and pharmaceutical sector, etc (Balan *et al*., 2012; Debashish *et al*., 2005).

The halophilic bacteria in the coastal regions are known to produce various enzymes that are important in industries (Haefner, 2003). The rhizospheric soil is the rich source of bacteria and other microbes (Hayat *et al*., 2010). The rhizospheric soil of plants growing on the Arabian beaches of Dakshina Kannada, Karnataka, India, was used in this investigation to extract halophilic bacterial strains. Arabian sea shoreline of Dakshina Kannada district stretches for about ~43 km, from Mulki to Thalappady according to Gazetteers, Government of Karnataka.

5.1 Isolation and Characterization of bacteria from the rhizosphere.

The sample collection region was the sea shore stretch from Sasihithlu beach in the north to Someshwara beach in the south (~36 km). The 36 km selected seashore stretch was evenly split into 6 km strata, and six sample collecting sites were chosen. Each sample collection site divided into 3 areas such as hundred meters, two hundred meters and three hundred meters from the coast. At all six sites, at 100 m, plants such as *Sporobolus pungens, Ipomoea purpurea* and *Rhizophora mangle* were found, at 200 m plants such as *Opuntia* and *Cocos nucifera* were found and at 300 m plants such as *Casuarina equisetifolia, Thespesia populnea* and *Ixora coccinea* were found. The plant *Scaevola plumieri* was found at 200 m only in two sites such as Sasihithlu beach and NITK beach.

Using the technique of serial dilution, a total of 90 halophilic bacterial isolates were isolated. Out of 90 isolates, the dominant and unique 32 isolates were selected for further screening for industrially important enzymes such as catalase, amylase, gelatinase, urease, oxidase etc., (Table 3.0). Out of 32 halophilic bacterial isolates, the isolates producing either gelatinase or catalase or both were identified based on 16S rRNA gene sequencing. Nine isolates identified as *Luteimonas marina* strain UCM1, *Ensifer sojae* strain UCM2, *Mesobacillus subterraneus* strain UCM3, *Cellulosimicrobium funkei* strain UCM4, *Rhizobium pusense* strain UCM5, *Bacillus subtilis* strain UCM6, *Citrobacter freundii* strain UCM7, *Bacillus cereus* strain UCM8, and *Microvirga* sp. strain UCM9 (Paramashree and Siddaraju, 2023).

5.2 Screening and Optimization of culture conditions for the beneficial microbes identified

The identified nine isolates were taken for further characterisation. They were tested for their growth against varied physiological parameters such as, pH, NaCl percentage (w/v) and temperature. The isolates were also tested for effect of carbon and nitrogen sources on their growth. The physiological parameters such as pH and temperature and nutritional factors including supplies of carbon and nitrogen were tuned to determine maximum growth of the isolates (Gupta *et al*., 2015).

5.2.1 The effect of physiological parameters on growth of the isolates

5.2.2 Effect of carbon sources on growth of isolates

Carbon sources are molecules that contain carbon and are used by organisms to synthesize biomass. Carbon is a vital element for bacteria, as it is used to synthesize carbon molecules that make up fats, proteins, nucleic acids and carbohydrates. Bacteria are able to utilize either organic carbon sources (sugars and alcohols) or inorganic carbon sources (carbon dioxide). Bacteria can be categorized into autotrophs or heterotrophs based on where they get their carbon. The only carbon source needed by autotrophs is carbon dioxide. Inorganic nutrients can be converted into organic molecules by an autotroph. Heterotrophs need carbon in organic forms. Heterotrophs are unable to convert inorganic nutrients into organic compounds (Bari and Yeasmin, 2022).

The majority of living things, including bacteria, may use a variety of substances as carbon sources. These substances can either be selectively employed with preference for the particular carbon sources chosen from among those that are accessible, or they can be co-metabolized (Shimizu, 2013b). The diauxie phenomenon, which was initially noticed by Monod (1942) in *E. coli*. This phenomenon in *E. coli* occurs when a combination of glucose and lactose is employed as a carbon source. Further research on this issue has shown that glucose is the preferred carbon source in many organisms (Shimizu, 2013b). Furthermore, using alternative carbon sources is frequently prohibited when glucose is present. Glucose repression, or more broadly, carbon catabolite repression (CCR), is the term used to describe this preference for glucose over other carbon sources (Magasanik, 1961; Shimizu, 2013b). The majority of heterotrophic bacteria, including facultatively autotrophic bacteria that suppress the genes for CO_2 fixation when an organic carbon source is present, exhibit CCR (Bowien and Kusian, 2002; Shimizu, 2013b). Certain potentially hazardous microbes, such Mycoplasma pneumonia and Chlamydia trachomatis, appear to lack CCR because they have evolved to thrive in nutritious host habitats (Halbedel *et al*., 2007; Shimizu, 2013b; Nicholson *et al*., 2004). Another condition involves,

utilization of glucose along with different carbon sources example, *Corynebacterium glutamicum* (Frunzke *et al*., 2008; Shimizu, 2013b; Wendisch *et al*., 2000). When preferable carbon sources are available, the genes for glucose utilization are repressed in certain bacteria, including *Pseudomonas aeruginosa*, *Bifidobacterium longum*, and *Streptococcus thermophilus*; this occurrence is referred to as reverse CCR (Parche *et al*., 2006; Shimizu, 2013b; Collier *et al*., 1996). In many bacteria, CCR is one of the most significant regulatory phenomena (Blencke *et al*., 2003; Moreno *et al*., 2001; Shimizu, 2013b; Liu *et al*., 2005). Cells need CCR in the competiting natural world, where choosing an ideal carbon source is essential to accelerating development and, ultimately, increasing survival rates. The production of virulent genes, which frequently allow the organism to make use of new nutrition sources, is another important function of CCR. The development of CCR may be used to choose the best carbon source for the most rapid development (Shimizu, 2013a; Shimizu, 2013b; Gorke and Stulke, 2008). Hence, it's important to characterize the bacteria about utilization of carbon source for its optimal growth.

In our study, the isolates were tested for their growth against some carbon sources such as, potato starch, lactose, maltose, and glucose.

Our study showed that, glucose was the best carbon source for optimum growth of *Luteimonas marina* strain UCM1, *Ensifer sojae* strain UCM2, *Mesobacillus subterraneus* strain UCM3, *Cellulosimicrobium funkei* strain UCM4 and *Bacillus subtilis* strain UCM6. The best carbon sources for optimum growth of *Bacillus cereus* strain UCM8 and *Microvirga* sp. strain UCM9 were both glucose and maltose. Potato starch was the best source for optimum growth of *Citrobacter freundii* strain UCM7. Lactose was the best source for optimum growth of *Rhizobium pusense* strain UCM5.

5.2.3 Effect of nitrogen sources on growth of bacterial isolates

Nitrogen is an essential nutrient for bacteria, and it's important to provide external nitrogen sources because it helps bacteria maintain their physiological and biochemical activity (He *et al*., 2023; Mobley *et al*., 2001). Bacteria can get nitrogen from organic sources, such as amino acids and nucleotide bases, or inorganic sources, such as ammonium sulfate, nitrates, urea, and ammonia (Mobley *et al*., 2001). Nitrogen is a building block for proteins, amino acids, ribonucleic acids, vitamins, and minerals. Additionally, it plays a crucial role in nitrogen metabolism, which forms the basis of the metabolism of microorganisms (He *et al*., 2023). Bacteria use complex internal regulatory networks to respond to different nitrogen environments. This helps them stay in an ideal state (He *et al*., 2023). Sources of nitrogen help bacteria grow

quickly and produce large amounts of cells (Chai and Adnan, 2018). Hence, it's important to characterize the bacteria against different nitrogen sources for its optimum growth.

We have characterized all nine identified bacteria against different nitrogen sources such as, urea, ammonium chloride, peptone, gelatin, yeast extract, skim milk powder, and beef extract to study their effect on growth of bacteria. Most of the studies used amino acids as nitrogen sources.

5.3 Screening and preliminary characterization of the halophilic enzymes.

Numerous economic domains could benefit from the hydrolytic enzymes found in hypersaline environments (Cojoc *et al*., 2009). Specific circumstances of the industrial process could render the enzymes inactive. The bacterium that can manufacture enzymes even in the lack of ideal conditions can be employed in this situation (Cojoc *et al*., 2009). According to our investigation, the identified nine isolates generate enzymes up to pH 11, 45 °C, and 8% NaCl (w/v), including catalase, oxidase, gelatinase and urease.

Urease is widely used in the process of biocalcification (Sarda *et al*., 2009), salt sedimentation, persistent human illness, etc., (Stabnikov *et al*., 2013; Konieczna *et al*., 2012). Catalase can be used to measure the amount of calcium in milk and water, treating acatalasemia, treating tissue damage and tumor metastasis, detecting bacterial infections that cause mastitis, and treating industrial waste (Ambarsari and Lindawati, 2017). Gelatinase used in the food, pharmaceutical, chemical industries and fundamental biological sciences. In the course of treating tumour metastasis, gelatinase plays a part in the breakdown of connective tissues. Gelatinase has many possible uses and is in great demand, thus research on novel strains, distinctive characteristics, and inexpensive, highly effective media preparations is becoming more and more interesting (Balan *et al*., 2012).

5.3.1 Gelatinase

Gelatinase is an extracellular metallo-endopeptidase or metalloproteinase that hydrolyzes collagen, casein, elastin, fibronectin, pheromone, gelatine, and proteoglycan (Balan *et al*., 2012; Mäkinen and Mäkinen, 1994, Mäkinen *et al*., 1989). Gelatinase ability to break down connective tissue linked to tumour spread makes it an essential ingredient in medication manufacture (Pacheco *et al*., 1998; Stetler-Stevenson *et al*., 1993). Pork, cow, and fish are the primary sources of gelatine used in the food, beverage, cosmetic, and pharmaceutical industries. To identify the different types of gelatine included in the products, several methods such as DNA analysis and protein-based analysis, were used. These techniques are not without limitations hence alternatively, scientists have revealed the gene expression of microbial gelatinase activity to

detect porcine gelatine (Shahimi *et al*., 2021). Studies have shown that animal hoofs, which are primarily composed of gelatine, may be degraded by bacterial (*Bacillus*) gelatinase (Mekonnen *et al*., 2022). The enzymatic hydrolysis of gelatine, improves the nutritional, functional, and textural properties. Gelatine hydrolysates have shown promise recently in a range of applications related to food composition (Liu *et al*., 2010). This is because these hydrolysates are high in pure protein, have a neutral flavor, and exhibit minimal bloom. As a result, it can be utilized to make protein drinks, smoothies, sweets, emulsifiers, and joint health nutritional items. It can also be used more broadly in the cosmetics sector (Liu *et al*., 2010).

Gelatinase has several possible uses, so it's important to look for new isolates that can produce gelatinase that is extremely effective, affordable, and resistant to extreme conditions (Balan *et al*., 2012). Although gelatinases are ubiquitous in nature, there aren't many discoveries originating from microbiological sources (Loperena *et al*., 2012; Barahona *et al*., 2016). Marine microorganisms are becoming the subject of increased research in the quest for biomolecules with commercial potential. Estimates indicate that coastal ecosystems are more biologically rich than tropical rain forests (Haefner, 2003).

In this study, we found that *Luteimonas marina* strain UCM1, *Ensifer sojae* strain UCM2, *Microvirga sp.* strain UCM9, *Bacillus cereus* strain UCM8, *Rhizobium pusense* strain UCM5, and *Cellulosimicrobium funkei* strain UCM4 produce gelatinase (Paramashree and Nanjundaiah, 2022).

The isolates, *Staphylococcus aureas*, *Pseudomonase aeraginosa*, *Clostridium perifergens*, and *Bacillus species* are among the bacteria that are employed in the industrial manufacturing of gelatinases (Mekonnen *et al*., 2018). The isolates of this study showed maximum gelatinase activity (in the range 26,000 U/mL – 57,000 U/mL) having extremotolerant property [pH 7 – 11 (maximum at pH 7, 8, 10, 11), 0 – 8% NaCl (w/v) (maximum at 2 – 3%) and 30 – 45 °C temperature (maximum at 30 – 40 °C)] which makes gelatinase enzyme extremely effective. All the isolates showed extracellular production of gelatinase enzyme, which makes purification of enzyme affordable. These unique properties make these isolates best source for industrial manufacturing of gelatinase.

5.3.2 Catalase

Enzyme catalase is an oxidoreductase enzyme which degrades hydrogen peroxide and acts as an antioxidant. One molecule of catalase can hydrolyse a million molecules of hydrogen peroxide. Catalase is a tetrameric protein having four chains of polypeptides, each containing

greater than 500 amino acids. It has four groups of porphyrin heme (iron), which enable the hydrogen peroxide to react (Kaushal *et al*., 2018). Catalase is reported to be active between pH 4 and pH 11 (Correia *et al*.,1994; Beers, 1961; Diaz *et al*., 2012; Calera *et al*., 2000) and all nine isolates of our study showed catalase activity at pH 7 – 11.

Catalase is used in different fields such as medical, reverting malignant features, clinical diagnosis, treatment of oxidative stress, food industry in food wrappers, catalase-glucose oxidase systems, production of cheese, determination of milk quality, bioremediation, hydrocarbon degradation, supply of oxygen for aerobic bioremediation, handling of textile industry bleaching effluents, biocorrosion of metals, and peroxide is eliminated from the contact lens solution (Beers and Sizer, 1952; Gromada and Fiedurek, 1997; Youn *et al*., 1995; Hussein, 2012).

Catalase is used as an oxygen provider in aerobic bioremediation. Hydrogen peroxide can be used as a substrate by catalase producing bacteria for the production of oxygen (Zappi *et al*., 2000). Some of the bacteria used as a source of catalase in bioremediation are, *Arthrobacter* sp. as indication of soil bioremediation's hydrocarbon degradation (Ogbolosingha *et al*., 2015), *Rhodococcus strain* to clean up contaminated crude oil (Leilei *et al*., 2012), *Bacillus SF* in textile bleaching wastewater, treatment and recycling (Fruhwirth *et al*., 2002; Paar *et al*., 2001), *Saccharomyces cerevisiae* and *Escherichia coli* in aerobic bioremediation (Sutton *et al*., 2011).

One of the most common illnesses affecting the livestock industry nowadays is infectious bovine mastitis. It causes changes in the composition of the milk that is obtained from the cow. To distinguish between the normal, healthy milk and the infected milk from the cow, one measure of milk quality that has been employed is the catalase test. This test is called Mastitis test. To conduct the test, fill the tube with 15 mL milk that will be examined, then add 5 mL of H_2O_2. Hydrogen peroxide is transformed into water and oxygen by the milk's catalase. The quantity of oxygen gathered serves as a gauge for the milk's quality; any quantity greater than 1.5 to 2.5 is deemed abnormal. A case of infectious mastitis is rarely missed by the catalase test unless the infection is nearly totally contained (Martin, 1979; Maddy, 1954).

In the production of cheese, especially Swiss cheese, cold pasteurization of milk is performed using hydrogen peroxide. All hydrogen peroxide must be eliminated from the milk because leftovers can interfere with the bacterial cultures needed for the actual cheese-making process. Usually, hydrogen peroxide is treated with catalase enzymes to produce water and molecular oxygen. They can be obtained from microbial sources or from the livers of cows. This is how the milk is treated after cold pasteurization to remove hydrogen peroxide and other

reactive oxygen species using the enzyme catalase (Geciova *et al*., 2002). It has been demonstrated that using catalase to eliminate excess hydrogen peroxide from milk samples used to manufacture cheese is a very cost-effective and environmentally responsible method (Kilcawley *et al*., 1989).

One of the most creative food packaging concepts to be brought about as a response to the ongoing shifts in consumer preferences and positive market trends is active packaging. Active packaging, also known as smart packaging, used to package food items, pharmaceuticals and numerous other items that need a longer shelf life (Kaushal *et al*., 2018). Substances that absorb oxygen are a major focus of active packaging techniques because they avoid oxidation of food products (Vermeiren *et al*., 1999). Food decay is frequently brought on by the food oxidation ingredients or mould deterioration. Food wrappers or packaging materials based on glucose oxidase-catalase can be used in the oxygen scavenging method. The highly specific enzyme glucose oxidase is found in the fungi *Penicillium* and *Aspergillus niger*, catalyses glucose oxidation by releasing hydrogen peroxide and moving two hydrogen atoms from the CHOH group of glucose to O_2. This produces glucono-1, 5-lactone, which uses molecular oxygen to hydrolyze spontaneously to gluconic acid. As a result, oxygen is utilized in this reaction and eliminated in the one that follows. The released hydrogen peroxide is degraded by catalase and glucose oxidase implicated in food wrappers. The degradation of hydrogen peroxide, protect food products (Cruz *et al*., 2012; Grigoras, 2017).

Our study showed that all the nine identified isolates showed catalase activity.

Different strains of *Luteimonas* such as, *Luteimonas zeaxanthinifaciens* sp. nov. strain YSD2104^T, *Luteimonas terricola* sp. nov. BZ92r^T, *Luteimonas aquatica* sp. nov., strain is RIB1-20^T, *Luteimonas composti* sp. nov. strain CC-YY255^T and *Luteimonas marina* sp. nov. JCM 12488^T showed catalase activity (Jung *et al*., 2024; Zhang *et al*., 2010; Chou *et al*., 2008; Baik *et al*., 2008). Our study presents new information that, *Luteimonas marina* strain UCM1 can produce catalase at pH 7 – 11, 0 – 2% NaCl (w/v) and 30 – 40 °C and strain UCM1 showed no effect on catalase activity against tested physiological parameters. Therefore, strain UCM1 showed extremotolerant catalase activity. The strain UCM1 showed maximum activity at pH 7, 2% NaCl (w/v) and 30 °C.

The species of genus *Ensifer* such as, *Ensifer Casida* 1982, *Ensifer Casida* 339VP, *Ensifer* sp. strain N7, *Ensifer adhaerens* sp. nov., strain A, *Ensifer meliloti* and *Ensifer meliloti* reported to have catalase activity (Young, 2003; Lei *et al*., 2009; Casida, 1982; Khalifa *et al*., 2018; Santos

et al., 2018). The isolate, *Ensifer adhaerens* NWODO-2 isolated from, Hogsback Forest reserve, South Africa, showed catalase activity of 2 – 6 U/mL at pH 5 – pH 9 (optimum, pH 7 i.e., 6 U/mL) (Falade *et al*., 2019). Whereas, more catalase activity seen in *Ensifer sojae* strain UCM2 at pH 7 - 11 in the range 34,833 - 23,733 U/mL respectively and highest at pH 9 i.e., 35,167 U/mL. The strain UCM2 had no changes in catalase activity at pH 7 – pH 9 and activity up to pH 11 is a new information. *Ensifer adhaerens* NWODO-2 reported to have catalase activity of 1 – 6 U/mL at 20 – 40 °C (optimum, 30 °C i.e., 6 U/mL) (Falade *et al*., 2019), this activity is very low compared to our study. The strain UCM2 showed catalase activity at 30 – 40 °C in the range 35,000 U/mL and 34,799 U/mL respectively and highest at 30 °C. The increase in temperature did not affect the catalase activity in strain UCM2. *Ensifer adhaerens* NWODO-2 reported to have catalase activity in yeast extract, ammonium chloride and ammonium sulphate in the range 4 – 12 U/mL and highest activity at combination of yeast extract and ammonium sulphate (Falade *et al*., 2019). The strain UCM2 also showed catalase activity in the presence of yeast extract. Our study presents new information that, *Ensifer sojae* strain UCM2 that it can show catalase activity at 0 – 5% NaCl (w/v). There was no changes in catalase activity at 2 – 4% NaCl (w/v). Our study also presents new information of catalase activity in strain UCM2 in the presence of carbon sources tested and ammonium chloride, urea, beef extract, peptone, and gelatine. The strain UCM2 showed maximum activity at pH 9, 2% NaCl (w/v) and 30 °C. Highest activity observed in wheat and gelatine.

The *Mesobacillus* species such as, *Bacillus subterraneus* sp. nov. strain COOI3B^T and *Virgibacillus subterraneus* sp. nov. strain H57B72^T showed catalase activity (Kanso *et al*., 2002; Wang *et al*., 2010). Our study presents new information that *Mesobacillus subterraneus* strain UCM3 has catalase activity at extreme physiological conditions such as, pH 7 - 11, 0 – 8% NaCl (w/v) and 30 – 45 °C. Strain UCM3 has also showed catalase activity in different carbon and nitrogen sources tested. The catalase activity was similar at pH 7 and pH 9. There was no changes in the catalase activity from 2 – 5% NaCl (w/v). The catalase activity was similar at 30 - 35 °C. The strain UCM3 showed maximum activity at pH 7, 2% NaCl (w/v) and 30 °C, highest in observed in wheat and yeast extract.

The isolate *Cellulosimicrobium funkei* and *C. funkei* strain AR8 showed catalase activity (Petkar *et al*., 2011; Karthik and Arulselvi, 2017). Our study presents new information that *Cellulosimicrobium funkei* strain UCM4 can show catalase activity at extreme physiological conditions such as, pH 7 - 11, 0 – 5% NaCl (w/v) and 30 – 40 °C. There was no changes in the

catalase activity at pH 7 and pH 8. There was a stable catalase activity at 1 - 4% NaCl (w/v). The strain UCM4 showed maximum activity at pH 7 and pH 8, 3% NaCl (w/v) and 30 °C.

The strains of *R. pusense* such as, *R. pusense* strain KHDEB5 and *Rhizobium pusense* MB-17a showed catalase activity (Das *et al*., 2021; Chaudhary *et al*., 2021). *Rhizobium pusense* GCB108 showed catalase activity at 3% NaCl (w/v) and *R. pusense* GCB117 showed catalase activity at 3 – 5% NaCl (w/v) (Nguyen *et al*., 2022). Whereas, *Rhizobium pusense* strain UCM5 of our study showed catalase activity at 0 – 5 % NaCl (w/v). There was no changes in the catalase activity against salinity. Our study presents new information that *Rhizobium pusense* strain UCM5, can show catalase activity at pH 7 – 11 and 30 – 40 °C temperature and also shows extremotolerant catalase activity. There was no changes in the catalase activity at pH 7 – pH 10. There was no changes in the catalase activity against temperature. The strain UCM5 showed maximum activity at, pH 9, 3% NaCl (w/v) and 30 °C.

It is reported earlier that, *B. subtilis* strains have produced catalase at varying yields, ranging from 3,500 U/mL to 39,117 U/mL (Jiang *et al*., 2023; Cao *et al*., 2014; Çalık *et al*., 2015; Yang *et al*., 2011), purified catalases from *B. subtilis* showed particular activity in the range of 1,140 U/mg to 55,784 U/mg (Jiang *et al*., 2023; Minning *et al*., 2001). *Bacillus subtilis* strain UCM6 also showed catalase between this reported range. Our study shows new information of *B. subtilis* strain UCM6, that it can produce extremotolerant catalase at pH 7 – 11, 0 – 8% NaCl (w/v) and 30 – 45 °C. There was no changes in the catalase activity at pH 7 – pH 10. There was no changes in the catalase activity at 2 – 4% NaCl (w/v). There was no changes in the catalase activity at temperature 30 – 40 °C. The strain UCM6 showed maximum activity at pH 9, 3% NaCl (w/v) and 30 °C.

Citrobacter freundii strain UCM7 showed more catalase activity compared to other strains. *Citrobacter freundii* (NRRL B-2643) which is reported to have 189.53 ± 9.72 U/mg of catalase (Salihu *et al*., 2024). Other strain of *C. freundii* such as, *Citrobacter spp.* and *Citrobacter freundii* str. nov haritD11 showed catalase activity (Nawaz *et al*., 2008; Meruvu *et al*., 2013). Our study shows *Citrobacter freundii* strain UCM7 can produce extremotolerant catalase i.e., at pH 7 – 11 0 – 8% NaCl (w/v) and 30 – 45 °C. There was no changes in the catalase activity at pH 7 and pH 8. There was no changes in the catalase activity at 0 – 5% NaCl (w/v). There was no changes in the catalase activity at 30 – 40 °C. The strain UCM7 showed maximum activity at, pH 7, 3% NaCl (w/v) and 30 °C.

Previous studies showed that, *Bacillus cereus* has catalase activity in the range 100 U/mL - 686.28 U/mL (Behera *et al*., 2014; Murthy *et al*., 2012), hence our study shows new information that *Bacillus cereus* strain UCM8 has 29,714 U/mL of catalase activity. Our study shows new information that, strain UCM8 can produce extremotolerant catalase activity i.e., at pH 7 – 11, 0 – 8% NaCl (w/v) and 30 – 45 °C. Its also new information that, strain UCM8 showed catalase activity in carbon sources and nitrogen sources tested. There was no changes in the catalase activity at pH 7 – pH 9. There was no changes in the catalase activity at 1 – 5% NaCl (w/v). There was no changes in the catalase activity at 30 – 35 °C. The strain UCM8 showed maximum activity at, pH 10, 5% NaCl (w/v) and 30 °C. Highest activity observed in wheat and yeast extract.

The species of *Microvirga* such as, *Microvirga soli* sp. nov. and *Microvirga thermotolerans* strain HR1 reported to have catalase activity (Dahal and Kim, 2017; Li *et al*., 2020). Our study shows new information that, *Microvirga sp.* strain UCM9, that it can produce extremotolerant catalase at pH 7 – 11, 0 – 2% NaCl (w/v) and 30 – 40 °C. It's also new information that strain UCM8 can produce catalase in the presence of carbon, and nitrogen sources tested. catalase activity was similar at pH 7 – pH 9. There was no changes in the catalase activity at 30 – 35 °C. The strain UCM9 showed maximum activity at, pH 8, 2% NaCl (w/v) and 35 °C. The catalase activity was highest in glucose and gelatine.

Other studies of different microorganisms on catalase activity are as follows; Halophilic archaeon *Halobacterium halobium* produces 10 - 20 U/mL of catalase up to 0 - 4% NaCl (w/v), 4.5 - 9.5 pH and 25 - 90 °C temperature (Brown-Peterson and Salin, 1993). *Aspergillus terreus* MTCC 6324 (Microbial Type Culture Collection stock culture (MTCC), Chandigarh produces catalase in the range 20,000,00 U/mL to 40,000,00 U/mL from pH 4 - 12.5 (highest at pH 11.5) and 25,000,00 U/mL to 95,000,00 U/mL of catalase at temperature range 25 – 95 °C (highest at 80 °C) (Vatsyayan and Goswami, 2016). *Acinetobacter* sp. YS0810 was identified from the Chinese coastal region of Qingdao, showed 6,818,89.741 U/mL of catalase activity. The catalase activity was detected in the pH range of 5 to 13, (retained 20% activity at extreme ends) and 10 - 80 °C temperature (retained 40 - 60% activity at extreme ends) (Fu *et al*., 2014). *Halobacterium halobium* showed catalase activity highest at pH 7.0 and remains active throughout a wide pH range of 6.5 to 10.0. This isolate was obtained from Mississippi, US state (Brown-Peterson and Salin, 1995)

5.3.2.1 Purification of catalase enzyme

There are only few studies available on purification of catalase enzyme from bacterial source. Till date, majority of the studies on enzyme synthesis have focused on terrestrial life, with a small number also studying marine organisms (Balan *et al*., 2012). Most of the bacteria produces intracellular catalase, hence it becomes difficult and takes many steps for the purification of catalase from the bacterial cell. Whereas, our study showed extracellular catalase enzyme production and the purification of catalase from *Bacillus cereus* strain UCM8 was performed in very simple steps. The partial purification of catalase from *Bacillus cereus* strain UCM8 is performed in simple steps such as, centrifugation of bacterial broth, ammonium sulphate precipitation to the supernatant, dialysis of the precipitated protein, Amicon filtration of dialyzed protein solution. Some bacteria showing extracellular catalase activity are, *Helicobacter pylori* (Ramarao *et al*., 2000), *Pseudomonas aeruginosa* (Pezzoni *et al*., 2014), *Exiguobacterium oxidotolerans* T-2-2T (Kiraly *et al*., 1997), *Vibrio rumoiensis* strain S-1 (Yumoto *et al*., 2000), *Staphylococcus aureus* ATCC 6538 P (Bukharin *et al*., 2002*), Anoxybacillus flavithermus* (Jaf *et al*., 2022), *Bacillus* sp. No.13 (Ogawa *et al*., 2004). The *Bacillus subtilis* GN99 isolated from food has reported show extracellular catalase activity and vegetative catalase protein purified was reported as 59.5 kDa of molecular mass (Naclerio *et al*., 1995).

5.3.2.2 The applications of catalase of this study

The crude oil effluents when discharged into soil, increase the soil pH to 8, which decreases the soil fertility, hence bioremediation of crude oil is essential to increase soil fertility. Studies have shown that the activity of catalase can be used for detection of bioremediation of crude oil in the soil (Kaushal *et al*., 2018). The microorganisms generally used as biomarkers, have limitations since they are inactive due to unfavourable conditions of the bioremediation process such as hypoxia, nutritional inaccessibility, and pH variations (Achuba *et al*., 2008). The bacterial isolates having extremotolerant growth property and also having catalase activity, at pH 8, can be used as a excellent alternative as a marker for bioremediation of crude oil instead of any other bacteria. These limitations are outstripped by the halotolerant bacterial isolates reported by us. Our study shows nine bacterial isolates with extremotolerant growth property and catalase activity from pH 7 to pH 11.

In the textile business, hydrogen peroxide is a frequently used bleaching chemical. Because of this, the textile industry's effluent contains a lot of hydrogen peroxide. When reducing H_2O_2, the use of chemicals like sodium bisulphite or hydro sulphite may cause an unintended

increase in the concentration of salt. As an alternative, H_2O_2 can be broken down by catalases. The degradation of hydrogen peroxide from textile effluent is effective by using catalase producing microorganisms. Catalase peroxidase (CP) extracted from *Bacillus* SF is utilized to handle textile bleaching wastewater (Gudelj *et al*., 2001). This isolate is reported to produce catalase, which is active at 50 °C temperatures and pH ranges of 6 - 10. The bacterial isolates of our study show catalase activity up to pH 11 and 45 °C, hence can be used as a better alternative in the bioremediation of textile effluent.

The two main sources of commercial catalase are *Aspergillus niger* and bovine liver (Singh *et al*., 2019). *Aspergillus niger* is used to make commercial catalases via a solid-state fermentation technique (Fiedurek and Gromada, 2000) for example, Catazyme® is the commercial catalase generated by an *Aspergillus niger* strain that has undergone genetic modification (Lambré *et al*., 2023; Catazyme®). *Aspergillus niger* strain 1102 showed stable catalase enzyme activity at pH 7 – pH 9 (optimum activity at pH 7) and optimum catalase activity at 50 °C (Yingqi *et al*., 2023). Whereas, our study outstripped this result i.e., our study shows isolates with catalase activity from pH 7 – 11 (optimum, 7 – 10). The fungi, *Aspergillus niger* typically requires 72 hrs for optimal growth and catalase production through solid state fermentation technique (Fiedurek and Gromada, 2000), on the other hand the isolates of our study show optimum growth and catalase activity at 12 – 17 hrs of incubation. The *Aspergillus niger* shows intracellular catalase activity (Fiedurek and Gromada, 2000) and compared to external enzymes, intracellular enzyme downstream processing is more complicated (Singh and Kumar, 2019). Which makes our study important since our study shows extracellular catalase activity hence purification of catalase protein was easier.

At the moment, *Escherichia coli* is the most widely utilized host for recombinant protein heterologous expression. *Bacillus subtilis*, *Bacillus anthracis*, and *Serratia marcescens* catalases have all been expressed in *E. coli*. (Jiang *et al*., 2023; Jia *et al*., 2016; Sooch *et al*., 2014; Ciofalo *et al*., 2006; Waterham *et al*., 1997). Cloning and recombinant expression of a catalase gene (Kat) from *Geobacillus* sp. CHB1 was done in *Escherichia coli* (E. *coli*). The purified recombinant catalase had a specific activity of 40,526 U/mg, and recombinant E. *coli* strain BL21 produced 35,831 U/mL of catalase, which was a high level. The recombinant catalase exhibited activity between pH 4 – pH 11 (optimal pH 6) and between 10 °C - 90 °C (optimum, 60 °C) (Jia *et al*., 2016). However, intracellular catalase synthesis in *E. coli* requires an expensive and time-consuming purification procedure. The recombinant protein heterologous expression and recombinant protein isolation and purification is a costly process (Jayakrishnan *et al*., 2024;

Kastberg *et al*., 2022; Merlin *et al*., 2014). Whereas, our study showed extracellular catalase enzyme production and the purification of catalase from *Bacillus cereus* strain UCM8 was performed in very simple steps. The partial purification of catalase from *Bacillus cereus* strain UCM8 in performed in simple steps such as, centrifugation of bacterial broth, ammonium sulphate precipitation to the supernatant, dialysis of the precipitated protein, Amicon filtration of dialyzed protein solution. Furthermore, the use of *E. coli* in food and medicine has been hindered by the safety concerns associated with the creation of endotoxins in this species (Jiang *et al*., 2023), these limitations can be outstripped by food grade isolates of our study. Also, *E. coli* can only grow up to 2.5 % NaCl (w/v) and further high salinity leads to degradation of *E coli* cells (Huang *et al*., 2001). This property makes *E. coli* not applicable in high saline experimental conditions. Whereas, our study showed bacterial isolates having catalase activity at 0 - 8% NaCl (w/v).

As an alternative to E. *coli* the yeast *Pichia pastoris* X-33 was used to express the catalase (KatA) from *Bacillus subtilis*. The gene was cloned and expressed in the yeast *Pichia pastoris* X-33 and produced 338.8 ± 9.6 U/mL of catalase in 2 days (Jiang *et al*., 2023). Whereas, our study showed maximum catalase activity (in the range 5,650 U/mL – 68,333 U/mL) with extremotolerant property [pH 7 – 11, 30 – 45 °C and 0 – 8% NaCl (w/v)]. The purified recombinant catalase from the yeast *Pichia pastoris* X-33 showed optimum activity at 25 °C and pH 11 (Jiang *et al*., 2023). Whereas, in our study, *Bacillus cereus* strain UCM8 showed maximum catalase activity at pH 10, 5% NaCl (w/v) and 30 °C and retained its 91% of activity at pH 11, 57% of activity at 8% NaCl (w/v) and 47% of activity at 45 °C. Additionally, the catalase's storage stability was found to be almost constant for 170 hrs at room temperature. At 4 °C, the activity of the enzyme slightly decreased, but after the sample was removed and left at room temperature for two hours, the activity returned to normal.

All these properties of the bacterial catalase of our study makes the isolates applicable in the fields of bioremediation, milk processing, textile industries and in food wrappers. with additional investigation, the halophilic bacterial isolates from our work could serve in future as a new source of highly effective, economically viable, extremotolerant enzymes for usage in relevant industries

To the best of our knowledge, the microbial diversity of the rhizospheric-halophilic bacterial population that produces industrially necessary enzymes has never been documented before, in Dakshina Kannada, Karnataka, India until now. (Paramashree and Siddaraju, 2023).

6.0 SUMMARY AND CONCLUSION

One type of extremophile that lives in saline conditions ranging between 0.8 - 8 M NaCl, are called halophiles (Oren, 2002b). Enzymes, proteins, biosurfactants, and other biomolecules found in halophiles are able to withstand a variety of adverse physiological circumstances, such as extreme salinity, extreme temperatures, a high pH, and dehydration (Singh and Singh, 2017). The unique properties of halophilic enzymes attracted researchers to this field of research (Karan *et al*., 2012). Biotechnology, polymeric materials, fermented pickles, biodegradation, biofuels, and other sectors are among the many applications for halophiles (Gupta *et al*., 2016; Oren, 2002b; Gomes *et al*., 2018; Chen and Jiang, 2018). Numerous studies have been conducted on halophilic bacteria from various hypersaline environments, examining their cellular changes, metabolisms, synthesis of enzymes, and activities. There is an absence of study on the wide range of halophilic bacteria that produce hydrolytic enzymes in different ecological situations. Hydrolysis is the first stage in the disintegration of complex organic compounds, and it significantly affects the nutrition metabolic cycle in saline environments. According to this viewpoint, research on halophilic bacteria is very important for environmental studies (Moreno *et al*., 2013). Numerous economic domains could benefit from the hydrolytic enzymes found in hypersaline environments (Cojoc *et al*., 2009). Specific circumstances that could render the enzymes inactive are necessary for the industrial process. The bacterium that can manufacture enzymes even in the lack of ideal conditions can be employed in this situation (Cojoc *et al*., 2009). To the best of our knowledge, no research has been done on halophilic bacteria throughout the Arabian coastal regions of Dakshina Kannada district, Karnataka state, India, and particularly not on rhizospheric halophilic bacteria in these regions. As a result, research and documentation of the rhizospheric halophilic bacteria in Dakshina Kannada's Arabian coastal regions is important.

In this study, we have isolated halophilic bacteria from the coastal regions of Dakshina Kannada, Karnataka, India. The rhizospheric soil of plants such as, *Sporobolus pungens*, *Ipomoea purpurea*, *Rhizophora mangle*, *Opuntia*, *Cocos nucifera*, *Casuarina equisetifolia*, *Thespesia populnea*, *Ixora coccinea* and *Scaevola plumieri* found growing along this coastal region, were collected. We have isolated ninety halophilic bacterial isolates found in the rhizospheric regions of selected plants. Out of ninety isolates, thirty-two prominent isolates were characterized by biochemical analysis and nine bacterial isolates which were producing enzymes catalase and gelatinase were identified based on 16S rRNA sequencing. The sequences were submitted to GenBank and accession numbers were obtained (Table 1.0). Phylogenetic tree of the identified

isolates was constructed using neighbor joining method. The nine isolates were identified as, *Luteimonas marina* strain UCM1, *Ensifer sojae* strain UCM2, *Mesobacillus subterraneus* strain UCM3, *Cellulosimicrobium funkei* strain UCM4, *Rhizobium pusense* strain UCM5, *Bacillus subtilis* strain UCM6, *Citrobacter freundii* strain UCM7, *Bacillus cereus* strain UCM8 and *Microvirga* sp. strain UCM9.

The effect of temperature, pH, starch-rich flours, carbon and nitrogen sources, and NaCl percentage (w/v) on the growth and enzyme activity of the isolates were analysed. The isolates were quantitatively analyzed for the production of catalase and gelatinase enzymes and also the impact of pH, NaCl (w/v), temperature against activity of these enzymes were quantified. The isolates, *Luteimonas marina* strain UCM1, *Rhizobium pusense* strain UCM5 showed gelatinase, catalase, oxidase, urease activity. Isolates, *Cellulosimicrobium funkei* strain UCM4, *Bacillus cereus* strain UCM8 and *Microvirga* sp. strain UCM9 showed gelatinase, catalase, oxidase activity. The isolates, *Ensifer sojae* strain UCM2 and *Citrobacter freundii* strain UCM7 showed, gelatinase, catalase, urease activity. Isolate, *Citrobacter freundii* strain UCM7 showed, catalase, oxidase, urease activity. The isolate, *Mesobacillus subterraneus* strain UCM3 showed, catalase, urease activity. The isolate, *Bacillus subtilis* strain UCM6 showed catalase activity. The isolates showed growth at pH range 7 – 11, temperature range 30 – 45 °C and 0 - 8 % NaCl (w/v).

The isolates showed extracellular gelatinase activity in the range 26,000 U/mL – 57,000 U/mL having extremotolerant property [pH 7 – 11 (maximum at pH 7, 8, 10, 11), 0 – 8% NaCl (w/v) (maximum at 2 – 3%) and 30 – 45 °C temperature (maximum at 30 – 40 °C)]. The isolates showed extracellular catalase activity in the range 5,650 U/mL – 68,333 U/mL having extremotolerant property [pH 7 – 11 (maximum at pH 7, 8, 9,10), 0 – 8% NaCl (w/v) (maximum at 2 – 5%) and 30 – 45 °C temperature (maximum at 30 – 40 °C)]. The isolates showed maximum catalase activity in glucose, lactose, maltose, mannitol and wheat as carbon source and gelatine, yeast extract as nitrogen source. The catalase activity of the isolates along the growth of isolates showed, that the catalase activity increased along the exponential phase, maximum catalase activity was seen at stationary phase (12 – 17 hrs of incubation) and the activity kept decreasing at decline and death phase. The catalase protein from, *Bacillus cereus* strain UCM8 was partially purified and the molecular weight was identified as ~ 50 – 56 KDa. The Lineweaver Burk plot for the purified catalase enzyme showed, Km = 0.022 and Vmax = 0.58.

The halophilic bacterial isolates of our study can be applied in different fields as a present good alternative. Gelatinase used in the food, pharmaceutical, chemical industries and fundamental biological sciences. In the course of treating tumour metastasis, gelatinase plays a

part in the breakdown of connective tissues (Balan *et al*., 2012). The extremotolerant property of gelatinase enzyme of our study make the isolates extremely effective. All the isolates showed extracellular production of gelatinase enzyme, which makes purification of enzyme affordable (Singh and Kumar, 2019). These unique properties make these isolates best source for industrial manufacturing of gelatinase. Catalase can be used to measure the amount of calcium in milk and water, treating acatalasemia, treating tissue damage and tumor metastasis, detecting bacterial infections that cause mastitis, and treating industrial waste (Ambarsari and Lindawati, 2017). The catalase of our study can be used as a marker of bioremediation of crude oil in the soil, bioremediation of textile industry effluents. For the commercial level of catalase production, *Aspergillus niger* is currently used (Singh *et al*., 2019). The bacterium, *Escherichia coli* is the most widely utilized host for recombinant protein heterologous expression of catalase (Jiang *et al*., 2023; Jia *et al*., 2016; Sooch *et al*., 2014; Ciofalo *et al*., 2006; Waterham *et al*., 1997). Both *Aspergillus niger* and *Escherichia coli* shows intracellular catalase activity hence, the downstream processing of intracellular enzymes is fairly complex as compared to extracellular enzyme (Fiedurek and Gromada, 2000; Jayakrishnan *et al*., 2024; Kastberg *et al*., 2022; Merlin *et al*., 2014; Singh and Kumar, 2019). Which makes our study important since our study shows extracellular catalase activity hence purification of catalase protein was easier. Studies have showed that, *Aspergillus niger* typically requires 72 hrs for optimal growth and catalase production through solid state fermentation technique (Fiedurek and Gromada, 2000). On the other hand, the isolates of our study show optimum growth and catalase activity at 12 – 17 hrs of incubation. *E. coli* can only grow up to 2.5 % NaCl (w/v) and further high salinity leads to degradation of *E coli* cells (Huang *et al*., 2001). These limitations are outstripped by bacterial isolates of our study, having catalase activity up to 8% NaCl (w/v).

With more investigation, the halophilic bacterial isolates from our findings might one day offer a new source of highly effective, economically feasible, and extremotolerant enzymes for use in related industries. To the best of our knowledge, this study is the first to outline the rhizospheric-halophilic microbial diversity that produces industrially significant enzymes in Dakshina Kannada, Karnataka, India.

7.0 BIBLIOGRAPHY

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ANNEXURE

LIST OF PUBLICATIONS

1. **Paramashree Deepa, B. K.,** and Nanjundaiah, S. M. (2022). Extremotolerant gelatinase enzyme production by UCM4 bacterial strain isolated from the rhizospheric soil of *Rhizophora mangle*. *Asian Journal of Biological and Life Sciences*, 11(*3*), 769-775. doi:10.5530/ajbls.2022.11.103.
2. **Paramashree Deepa, B. K.,** and Siddaraju M. N. (2023). Isolation and identification of halophilic bacteria associated with rhizospheric soil from the Arabian coast of Dakshina Kannada, Karnataka, India. *Journal of the Marine Biological Association of India*, 65(*1*), 17-29. doi:10.6024/jmbai.2023.65.1.2388-02.

PRESENTATIONS IN NATIONAL AND INTERNATIONAL CONFERENCES

- poster presentation on "Extremophiles and biotechnology: Current trends and applications". One day National conferences on "Current Advances in medical and industrial biotechnology". Alva's College, Mangalore, 20th February 2020.
- Participated and submitted abstract "The study of rhizospheric bacteria of halophilic plants for potential enzyme production" international E-Conference on "Advances and Future Outlook in Biotechnology and Crop Improvement for Sustainable Productivity". Department of Biotechnology & Crop Improvement College of Horticulture, UHS Campus, GKVK Post, Bengaluru, 24-27th November 2020.
- Oral presentation on "A study on production of industrially important enzymes from extremophilic bacteria". International conference on Advances in Biosciences and Biotechnology (ICABB-2022). Jayeep Institute of Information Technology, Noida, 20-20th January 2022.

- Oral presentation on “Enzymatic study and industrial application studies of extremophilic bacteria”. National Conferences MICON 2022 – “Advances in Microbiology”. Yenepoya Research Centre, Mangalore, 2nd February 2022.